DISCUSSION OF FIVE YEARS' USE OF DDT RESIDUALS AGAINST *ANOPHELES QUADRIMACULATUS*

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Five years of operational work have been completed on the DDT Residual Spray Program for malaria control which was begun in 1945 by state departments of health in the southeastern United States in cooperation with the Communicable Disease Center of the Public Health Service. This program consists of applying residual coatings of DDT to the interiors of houses and privies and is based upon the principle of specific anopheline mosquito control. During the first two years of operation this program was termed the Extended Malaria Control Program to distingush it from the war-time Malaria Control in War Areas (MCWA) program which was concerned with the protection of military personnel from indigenous malaria in the United States, whereas, the Extended Program functioned to protect the civilian population from imported foreign malaria following the close of the war. Because of the success of the Extended Program as evidenced by a continuing precipitous decline in malaria morbidity and mortality, Federal funds were forthcoming to assist states in undertaking a five-year Malaria Eradication Program (Andrews and Gilbertson, 1948). This Malaria Eradication Program was launched on July 1, 1947. In that year residual spray operations were expanded to cover 326 counties and included the spraying of approximately 900,000 homes with DDT. With further increases in local financial participation by the states it was possible in 1949 to bring the number of operating counties to 344 and the number of homes sprayed to almost one million.

The reduction of the malaria hazard by the Residual Spray Program has been measured during the past five years in terms of its ability to maintain houses free of *Anopheles quadrimaculatus*. Control operations in this country have been directed specifically against those individual malaria mosquitoes which enter houses to bite and therefore, are most likely to transmit malaria. The *A. quadrimaculatus* habit of resting and lingering within houses, especially after a blood meal, is well-known; and it is this characteristic which results in control through the inside residual spraying of houses. Conclusions as to the effectiveness of the Residual Spray Program are based on data secured from inspections of randomly selected sprayed and unsprayed houses for the presence or absence of *A. quadrimaculatus*.

A five-year summary of data is given in table 1 covering the entomological evaluation of control operations on the Residual Spray Program for 13 Southeastern States during the years 1945 through 1949. The data are based on more than 66,000 inspections of sprayed and unsprayed houses. It should be noted that in 1945, the first year of the program, only sprayed houses were inspected together with natural resting places such as barns and privies; inspections of unsprayed houses for comparative purposes were not made until 1946 (Bradley, 1946). In 1945 two seasonal applications of DDT were applied at the rate of 100 milligrams per square foot. However, evaluation data indicated that a single seasonal application of 200 mil-

ligrams per square foot would be about equally effective from the standpoint of long-lasting residual and at the same time would be operationally more economical. Consequently, in subsequent years, 1946 through 1949, the 200 milligram application rate has been recommended and used in nearly all states.

It may be observed in table 1 that the average percentage of sprayed houses maintained free of *A. quadrimaculatus* for the five-year period, 1945–1949, was 98.3 as compared to only 84.0 for unsprayed houses. Thus, the indicated over-all control (reduction in houses infested) was approximately 90 per cent for the five-year period. The gradual decrease in effectiveness of the residual during the course of the season is indicated by the average percentage of houses free of *A. quadrimaculatus* for each successive month following the spray application. During the first month after

TABLE 1

Five-year summary of entomological evaluation of residual spray program in 13 southeastern states, 1945–1949, based on 66,007 inspections of sprayed and unsprayed houses

Reduction in malaria hazard through residual DDT spraying is indicated by comparison of percentages of inspected houses found free of *Anopheles quadrimaculatus* in the afternoon.

MONTHS AFTER SPRAYING	NO. OF HOUSES INSPECTED						PERCENTAGE OF HOUSES FREE OF *A. quad.* IN P.M.					
	1945	1946	1947	1948	1949	Total	1945	1946	1947	1948	1949	Ave. 1945–1949
Sprayed houses												
0–1	3916	6018	1546	699	1069	13,248	98.9	99.2	99.2	96.9	99.6	99.0
1–2	4558	6739	2690	1354	1999	17,340	98.3	99.0	98.5	98.2	98.8	98.7
2–3	3557	5321	2538	2093	2583	16,092	95.7	99.1	98.7	98.2	98.7	98.1
3–4	1375	2974	1578	2102	1977	10,006	94.7	98.7	98.7	97.3	98.8	97.9
4–5	723	899	442	1231	885	4,180	94.2	98.2	98.9	94.6	99.1	96.7
Total.....	14,129	21,951	8,794	7,479	8,513	60,866	97.2	99.0	98.8	97.2	98.9	98.3
Unsprayed houses												
	—	1,639	1,170	1,021	1,311	5,141	—	87.3	72.0	83.3	91.2	84.0

spraying, 99.0 per cent of sprayed houses were free from *A. quadrimaculatus*; during the second month only 98.7 per cent; in the third month 98.1 per cent; for the fourth month 97.9 per cent, and for the fifth month 96.7 per cent. The indicated percentages of control, for successive periods 0–1, 1–2, 2–3, 3–4, and 4–5 months after spraying were 94, 92, 88, 87, and 79 per cent, respectively. Thus, it seems apparent that only a relatively gradual decrease in effective control occurs during the first 4 months after application, but thereafter the decline in effectiveness is accelerated.

Table 2 presents a summary of data on evaluation of the program for the years 1946 through 1949, showing for each year the ratio of the number of sprayed and unsprayed houses in which *A. quadrimaculatus* were found, to the total number of sprayed and unsprayed houses inspected, and the indicated percentages of control

obtained for the several years. The reason for omitting the year 1945 has already been given.

A comparison of the percentages of sprayed houses maintained free of *A. quadrimaculatus* for the years 1946 through 1949 (table 2) shows that there is a small but significant amount of difference from year to year in these figures. A more marked degree of variation is seen in the comparable percentages for unsprayed houses. Two points appear worthy of emphasis here. One is that the variation from year to year between the percentages of unsprayed houses free from mosquitoes is believed to reflect the annual variations which occur in mosquito abundance from year to year in the Southeastern States. In other words, it is indicated that the mosquito crop in 1947, when only 72.0 per cent of the unsprayed houses were free of A. *quadrimaculatus*, was considerably larger, for example, than in 1949 when this percentage was 91.2. The other point is that the small but significant variation in the percentages of sprayed houses which were free from mosquitoes indicates the relatively consistent high-level of control being achieved by the application of residual DDT spray. That this is true may be further illustrated by examining the indicated percentages of control achieved in the different years, namely, 92.1 per cent in 1946; 95.7 per cent in 1947; 83.2 per cent in 1948; 88.5 per cent in 1949, and by the over-all average for this four-year period of 91.9 per cent control. These calculated percentages of control take into consideration the relative difference in mosquito abundance for the different years and they seem to demonstrate, other factors being equal, that effective control may be realized irrespective of seasonal variations in mosquito abundance.

TABLE 2

Condensed summary of entomological evaluation of residual spray program, 1946–1949

	1946	1947	1948	1949	AVERAGE 1946–49
Sprayed					
(1) Percentage of Houses Free of *A. quadrimaculatus*	99.0	98.8	97.2	98.9	98.7
(2) Ratio of: No. Houses with *A. quadrimaculatus* to No. Houses inspected	1:100	1:80	1:36	1:91	1:74
Unsprayed					
(3) Percentage of Houses Free of *A. quadrimaculatus*	87.3	72.0	83.3	91.2	84.0
(4) Ratio of: No. Houses with *A. quadrimaculatus* to No. Houses Inspected	1:8	1:4	1:6	1:11	1:6
(5) Indicated Percentage of Control	92.1	95.7	83.2	88.5	91.9

Another way of showing, possibly a little more clearly, the magnitude of variation in annual malaria mosquito populations from year to year is by comparing the annual ratios of the number of unsprayed houses inspected in which mosquitoes were found

to the total number of unsprayed houses inspected (table 2). These ratios are as follows: 1949, 1:11; 1948, 1:6; 1947, 1:4; and 1946, 1:8. Thus, in the year 1947, when mosquitoes are considered to have been most abundant, they were found in one house in every four inspected, while in the year 1949 when mosquitoes were much less numerous, only one house out of every eleven inspected harbored mosquitoes. Similarly determined ratios for sprayed houses for these same years are worthy of note because they show by comparison the high degree of control realized on the Residual Spray Program. These ratios are as follows: 1949, 1:91; 1948, 1:36; 1947, 1:80; 1946, 1:100. The ratio of 1:36 for the year 1948 appears to be a radical departure from the ratios for the other three years and is of especial interest to us from an evaluation of control standpoint. The calculated percentage of control realized in 1948 was only 83.2 as compared with the average percentage of 91.9 for the four-year period, 1946–1949, or the average percentage of 93.7 for the three years 1946,

TABLE 3

Deviation from expectation of observed and expected sprayed and unsprayed houses with A. quadrimaculatus, *1946–1949*

YEAR	DEVIATION FROM EXPECTATION	OBSERVED AND EXPECTED NUMBERS					
		Number of Houses Inspected		Number of Houses With *A. quadrimaculatus*			
				Sprayed		Unsprayed	
		Sprayed	Unsprayed	Obs.	Exp.	Obs.	Exp.
1949	21.3	8513	1311	94	72.7	116	137.3
1948	54.3	7479	1021	206	151.7	170	224.3
1947	−70.2	8794	1170	109	179.2	328	257.8
1946	−5.4	21951	1639	220	225.4	208	202.6

1947, and 1949. This reduction in effective control during 1948 quite possibly is accounted for by the fact that in this year a certain amount of difficulty was experienced through the use of substandard spray formulations in some areas. That this is a plausible explanation is supported by the fact that in each of the years 1947 and 1949 a much higher degree of mosquito control was obtained. It also is worthy of note that in the former year, 1947, mosquito populations were at the highest of any year during the period being reported.

Assuming that the entomological data presented here are a comparable measure of mosquito densities from year to year, it appears that annual differences in the percentages of control (table 2) were of statistical significance and that control operations were most effective in 1947, with 1946 ranking second, 1949 third, and 1948 in the lowest position.

The probability of obtaining by chance alone the observed difference in relative percentage changes between sprayed and unsprayed houses from year to year may be calculated (Norton, 1945) by computing the expected numbers of mosquitoes in sprayed and unsprayed houses each year on a hypothesis of homogeneity, and then

using the chi-square distribution to estimate the chance of obtaining the observed interaction by chance alone.

Table 3, based upon Norton's iterative method of calculating deviations from expectation, shows the deviation for each year, together with the observed and expected numbers of sprayed and unsprayed houses with mosquitoes.

By computation, the observed value of chi-square is 99.5. For three degrees of freedom the chance of obtaining a chi-square of 21.2, or larger, is only .0001. Therefore, it is very unlikely that the changes from year to year in relative percentages of houses harboring *A. quadrimaculatus* in sprayed and unsprayed areas would arise by chance alone. Thus, the number of sprayed houses in which *A. quadrimaculatus* were found each year, allowing for change in unsprayed houses, departed from expectation for the four-year period in the following manner:

1949 Somewhat above the average
1948 Markedly above the average
1947 Markedly below the average
1946 Very near the average

Statistical evidence supports, therefore, the indicated percentages of control given in table 2.

SUMMARY AND CONCLUSIONS

1. A summary of the results of five years (1945–1949) of operat onal work using residual DDT for the control of *A. quadrimaculatus* in the southeastern United States, shows that effective control of malaria mosquitoes has been achieved Of the sprayed houses inspected, 98.3 per cent were found free of *A. quadrimaculatus* as compared with 84.0 per cent for unsprayed houses. Thus, the average indicated control was approximately 90 per cent for the five-year period.

2. The greatest degree of control (95.7 per cent) was obtained in 1947, a year when mosquitoes were considered to have been most abundant, since one in every four unsprayed houses inspected harbored mosquitoes. The lowest degree of control (83.2 per cent) was obtained during 1948 which is attributed to the use of substandard DDT formulations in some areas during that year.

3. A gradual decrease in effectiveness of residual DDT occurred during the course of the season. This is indicated by lower average percentages of houses free of malaria mosquitoes for each successive month following the spray application.

4. A statistical treatment of the data presented shows that from year to year significant differences occurred between the relative number of sprayed and unsprayed houses in which malaria mosquitoes were found and supports the conclusion that real differences occurred in the percentage of control as calculated for each of the several years.

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RESUMEN Y CONCLUSIONES

1. Un resúmen de los resultados obtenidos durante cinco años (1945–1949) mediante el uso de DDT residual para el control de *A. quadrimaculatus* en el Sureste de los Estados Unidos indica que se ha alcanzado control efectivo de los mosquitos transmisores de malaria. El 98,3 por ciento de las casas rociadas fueron negativas a *A. quadrimaculatus*, contra el 84,0 por ciento entre las casas no rociadas. Por consiguiente el control promedio durante el período de los cinco años fué aproximadamente del 90 por ciento.

2. El más alto grado de control (95,7 por ciento) fué obtenido en 1947, un año de gran abundancia de mosquitos puesto que una por cada cuatro casas rociadas inspeccionadas acusó presencia de mosquitos. El más bajo grado de control (83,2 por ciento) fué obtenido durante el año de 1948 lo que se ha atribuído al uso de fórmulas de DDT por debajo de los standardsen algunas zonas durante aquel año.

3. Una baja gradual en la efectividad del DDT residual ocurrió durante la estación. Esto es indicado por el descenso en los porcentajes promedios de casas libres de mosquitos de malaria en cada mes sucesivo siguiente al del rociado.

4. Un tratamiento estadístico de los datos presentados muestra que de año en año ocurren diferencias significantes entre los números relativos de casas rociadas y no rociadas en las cuales se hallaron mosquitos de malaria y sustenta la conclusión de que ocurren diferencias reales en el porcentaje de control para cada una de los diferentes años.

FURTHER OBSERVATIONS ON THE DEVELOPMENT OF SPOROZOITES OF *PLASMODIUM GALLINACEUM* INTO CRYPTOZOITES IN TISSUE CULTURE[1]

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In a previous paper we reported the development of sporozoites of *Plasmodium gallinaceum* into cryptozoites in tissue cultures of normal chicken macrophages (Dubin, Laird and Drinnon 1949). These earlier experiments were run for only two to three days. Since then we have found that the dosage[3] of antibiotics used in the previous experiments resulted in loss of the cultures by infection at the end of three to four days. For this reason further experiments were done in which both the concentrations of antibiotics and the schedule of treating the cultures were modified. This resulted in a procedure which permitted maintaining the cultures free of infection up to seven days. Following this, chicks were infected with such pre-erythrocytic stages grown *in vitro* for several days. In addition, this paper reports unsuccessful attempts to duplicate these results in studies with human malaria.

I. Attempt to maintain cultures bacteria-free longer than 48 hours with previous dosage of antibiotics

Following our initial success in obtaining the development of sporozoites into cryptozoites *in vitro*, we attempted to maintain such cultures for longer periods of time, in order to inoculate chicks with cultures, the age of which would be considerably beyond the survival time of the originally inoculated sporozoites.[4] This was attempted by using 100 units of penicillin G (Squibb) and 400 microgram-equivalents of dihydrostreptomycin base (Squibb) per ml.[5] in the nutrient medium in which the sporozoites were inoculated, followed by incubating the cultures undisturbed for 48 hours. At 48 hours the old nutrient was removed and replaced by fresh nutrient containing identical amounts of antibiotics. The cultures were examined at 72 and 96 hours respectively. At 96 hours all cultures were almost completely over-run and destroyed by bacteria. Many cultures were badly damaged by bacterial growth at 72 hours. No cryptozoites were seen in these cultures. Apparently the concentration of antibiotics had reached a low enough level by 48 hours so that some bacteria sur-

[1] A contract with the Office of Naval Research, Microbiology Branch, Number N8onr-75700, and cooperation of the Tennessee Valley Authority through financial assistance provided under contractual agreement have made this work possible.

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[3] For the purpose of this communication we are using the word "dosage" to mean a regular schedule of adding definite amounts of antibiotics to the tissue culture medium.

[4] Mr. Harvey Akins of the Laboratory of Tropical Diseases of the Public Health Service in Memphis kindly furnished the *Aedes aegypti* mosquitoes infected with *P. gallinaceum*.

[5] In the remainder of the paper, "units of penicillin G" will be abbreviated to "u P" and "microgram-equivalents of dihydrostreptomycin base" will be shortened to "m S".

vived, and these were able to multiply in spite of the further addition of antibiotics at 48 hours. It seemed desirable, therefore, to determine the rates of deterioration of penicillin and streptomycin.

II. Rates of deterioration of antibiotics

The rates of deterioration of the antibiotics were studied under conditions of temperature, hydrogen-ion concentration and concentration of nutrient similar to those of the experimental conditions, but in the absence of cells and bacteria.[6] This was done separately for penicillin and dihydrostreptomycin. Culture flasks were set up containing 25 per cent serum in Tyrode's solution with 0.0025 per cent of phenol red added. One group contained 100 uP per ml., while the other contained 400 mS per ml. The flasks were incubated for 48 hours at 37° C. and at pH 7.4 controlled by continuous flow of 2 per cent CO_2 in air. Aliquot samples for assay were taken just prior to incubation, at 24 hours and at 48 hours, respectively. The methods for estimation of penicillin and streptomycin were those described by Randall, Price and Welch (1945) and Price, Nielsen and Welch (1946) respectively. At 24 hours the concentration of penicillin was 60 per cent of the original value and that of dihydrostreptomycin 80 per cent of the original value. At 48 hours the concentrations of penicillin and dihydrostreptomycin were each about 50 per cent of the original value. Since these studies were made in the absence of metabolizing cells and bacteria, these rates of deterioration probably represented minimum rates.

On the basis of these results it was decided to maintain a more constant level of antibiotics by adding further amounts to the flask at 24 hours without removing the nutrient and to follow this by changing the entire nutrient at 48 hours. This was considered preferable to changing the nutrient every 24 hours, which would result in considerable loss of cells and parasites. Accordingly, a schedule was set up whereby fresh nutrient containing appropriate amounts of antibiotics was placed in the flask at zero and 48 hours and further amounts of antibiotics added at 24 and 72 hours without removal of nutrient. These additional amounts, which represented about 50 per cent of the concentrations of the antibiotics present at zero hours, were added to each flask in 0.05 cc. of modified glucosol. Since each flask contained about one cc. of nutrient, this additional volume represented only 5 per cent of the original volume. This degree of dilution of serum and bicarbonate was considered negligible.

III. Use of higher concentrations of antibiotics

Because the use of the previous dosage resulted in loss of the cultures by bacterial contamination it was decided to increase the concentrations of antibiotics in the next group of experiments. This group of experiments is summarized in Table 1. The rinsing solution contained 1000 uP and 600 mS per ml. The nutrient contained 1000 uP per ml. together with concentrations of dihydrostreptomycin varying from 200 m to 800 m per ml.

In all of these flasks, there was complete inhibition of bacterial growth, as well as

[6] These determinations were made by Miss Mary E. Cowan of the Department of Pathology and Bacteriology, University of Tennessee, Memphis.

TABLE 1

Effects of high concentrations of penicillin and dihydrostreptomycin in combination on bacteria, macrophages and on the development of sporozoites into cryptozoites in tissue culture

EXPERIMENTAL GROUP	CONCENTRATION OF ANTIBIOTICS PER ML.						RESULTS		
	Rinsing Solution	Dosage					Cell Growth	Cryptozoites	Bacteria
		0 hrs.	24 hrs.	48 hrs.	72 hrs.	96 hrs.			
Group 3	1000 P 600 S	1000 P 400 S F	500 P 200 S A	1000 P 400 S F	500 P 200 S A	Cultures fixed	$\frac{3}{5}$ flasks no growth; $\frac{2}{5}$ flasks poor growth	0	0
Group 2	1000 P 600 S	1000 P 400 S F	1000 P 400 S F	1000 P 400 S F	Cultures fixed		$\frac{1}{4}$ flasks no growth; $\frac{3}{4}$ flasks moderate growth	0	0
Group 1	1000 P 600 S	1000 P 200 S F	500 P 100 S A	1000 P 200 S F	Cultures fixed		$\frac{1}{4}$ flasks no growth; $\frac{3}{4}$ flasks moderate growth	0	0
Group 4	1000 P 600 S	1000 P 800 S F	500 P 250 S A	1000 P 800 S F	Cultures fixed		$\frac{1}{4}$ flasks no growth; $\frac{3}{4}$ flasks very poor growth	0	0

F—Fresh nutrient added after old nutrient discarded.
A—Addition of antibiotics without change of nutrient.

absence of cryptozoites and damage to the macrophages. The damaging effect on the macrophages was especially marked in the experimental group which was treated with 1000 uP and 800 mS per ml.

These results indicated that while these higher concentrations of antibiotics completely inhibited bacterial growth, the use of such concentrations was impracticable since this resulted in considerable damage to macrophages and complete absence of cryptozoites.

IV. Studies on effects of penicillin and streptomycin in combination on cultures of exoerythrocytic stages of P. gallinaceum *in vitro*

The results of the experiments described in the previous paragraph were somewhat surprising since the original work indicated that the macrophages and parasites could tolerate up to 5000 uP per ml. or 400 mS per ml. when these antibiotics were used separately. These results suggested, therefore, that there was a synergistic action when the antibiotics were used in combination. Consequently, we went back to studies on the effect of the antibiotics in combination on the exoerythrocytic stages of *P. gallinaceum* grown from infected chick embryo spleen. Five experimental groups were set up, in addition to the control group (Table 2). The concentrations in the first group consisted of 100 uP and 50 mS per ml. and those in the second group 100 uP and 100 mS per ml. The results of test groups 1 and 2 were similar to those of the controls. In test group 3, however, which contained 100 uP and 200 mS per ml., while there was no evidence of cell damage there was a suggestion of a slight reduction in the number of parasites as compared with the control group. In test group 4, which contained 100 uP and 400 mS per ml., the cells were somewhat reduced in number and, in addition, showed a peculiar elongated character. In this group also parasites were reduced in number to about one-half of that of the control group. In group 5, which contained 1000 uP and 400 mS per ml., the cells showed evidence of considerable damage. They were reduced in number to about one-third of that of the control group and showed a peculiar elongated or shrunken appearance. In addition many cells contained a bluish-staining precipitate in the cytoplasm. In this group, also, the parasites were reduced in number to about one-fourth of that of the control group. It was evident from these results that penicillin and streptomycin when used in combination had a synergistic damaging effect on the cells and parasites. In later studies, therefore, it was decided to stay below a level of a combination of 100 uP and 100 mS per ml.

V. Use of lower concentrations of antibiotics

In this group of experiments the methods were similar to those described above except for differences in concentrations of antibiotics (Table 3). The rinsing solution contained 100 uP and 100 mS per ml. In test group 1 the nutrient at zero hours contained 50 uP and 50 mS per ml., and at 24 hours the concentration of antibiotics was augmented by the addition of 25 uP and 25 mS per ml. In group 2 the nutrient at zero hours contained 100 uP and 100 mS per ml. and the addition at 24 hours consisted of 50 uP and 50 mS per ml. In group 3 the nutrient at zero hours contained 500 uP and 100 mS per ml. and the addition at 24 hours consisted of 200 uP and 50

TABLE 2

Effects of penicillin and dihydrostreptomycin in combination on macrophages and exoerythrocytic forms of P. gallinaceum *grown in tissue culture from infected chick embryo spleen*

EXPERIMENTAL GROUP	DOSAGE (CONCENTRATION OF ANTIBIOTICS PER ML.)					RESULTS	
	0 hrs.	24 hrs.	48 hrs.	72 hrs.	96 hrs.	Growth of Macrophages*	Parasites†
Control Group	0	0	0	0	Cultures fixed	4+	4+
Test Group 1	100 P 50 S F	50 P 20 S A	100 P 50 S F	50 P 20 S A	Cultures fixed	4+	4+
Test Group 2	100 P 100 S F	50 P 40 S A	100 P 100 S F	50 P 40 S A	Cultures fixed	4+	4+
Test Group 3	100 P 200 S F	50 P 80 S A	100 P 200 S F	50 P 80 S A	Cultures fixed	4+	3+
Test Group 4	100 P 400 S F	50 P 160 S A	100 P 400 S F	50 P 160 S A	Cultures fixed	3+ (cells unusually elongated)	2+
Test Group 5	1000 P 400 S F	500 P 160 S A	1000 P 400 S F	500 P 160 S A	Cultures fixed	1 to 2+ (cells elongated, shrunken and contained bluish precipitate)	1+

F—Fresh nutrient added after old nutrient discarded.
A—Addition of antibiotics without change of nutrient.
* Growth of macrophages was estimated quantitatively in terms of the area of coverslip covered by the cells.
† Number of parasties was estimated in terms of number of parasites per unit area of coverslip.

mS per ml. The results were similar in all three test groups. At 72 hours there was an excellent growth of macrophages with no evidence of cell damage. There were no or rare bacteria and fungi in the flasks, and in all groups the flasks contained cryptozoites. Consequently, in all future experiments the following schedule was used: (a) the rinsing solution contained 100 uP and 100 mS per ml., (b) on even days fresh nutrient was used containing 100 uP and 100 mS per ml., and (c) on odd days additions were made of 50 uP and 50 mS per ml. This schedule was used successfully in obtaining cultures of macrophages and pre-erythrocytic stages, free of bacteria, up to seven days of incubation after the inoculation of the sporozoites.

TABLE 3

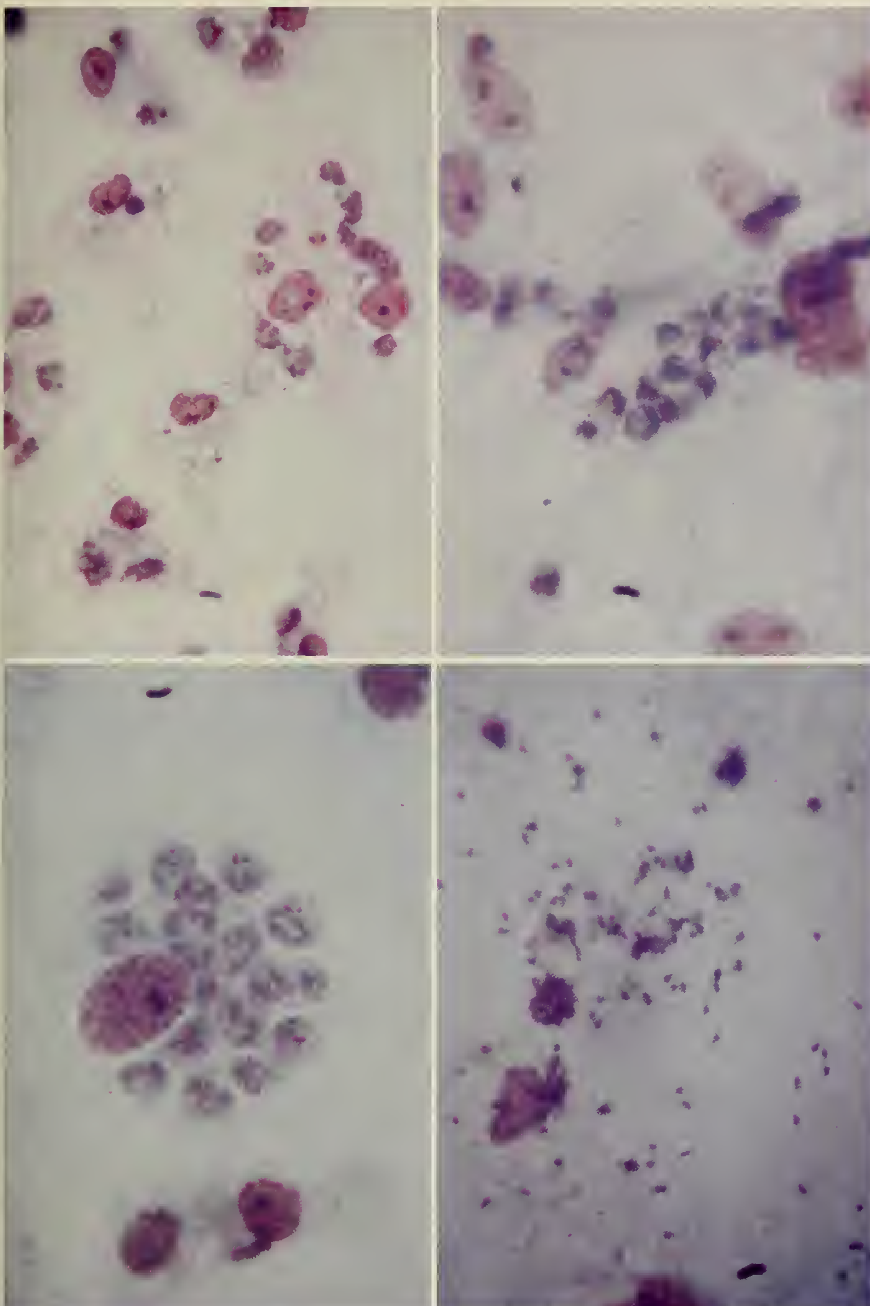
Effects of lower concentrations of penicillin and dihydrostreptomycin in combination on bacteria, macrophages and on the development of sporozoites into cryptozoites in tissue culture

EXPERIMENTAL GROUP	CONCENTRATION OF ANTIBIOTICS PER ML.					RESULTS		
	Rinsing Solution	Dosage				Cell Growth	Cryptozoites	Bacteria
		0 hrs.	24 hrs.	48 hrs.	72 hrs.			
Group 1	100 P 100 S	50 P 50 S F	25 P 25 S A	50 P 50 S F	Cultures fixed	Excellent	Present	None or rare
Group 2	100 P 100 S	100 P 100 S F	50 P 50 S A	100 P 100 S F	Cultures fixed	Excellent	Present	None or rare
Group 3	100 P 100 S	500 P 100 S F	200 P 50 S A	500 P 100 S F	Cultures fixed	Excellent	Present	None or rare

F—Fresh nutrient added after old nutrient discarded.
A—Addition of antibiotics without change of nutrient.

VI. Observations on cultures of pre-erythrocytic stages of P. gallinaceum *grown for seven days*

In one experiment cultures were incubated for seven days after inoculation of the sporozoites. These cultures were free of bacteria, but occasional fungi were seen. These fungi, however, did not appear to interfere with the cultures of macrophages or metacryptozoites present in the flasks. While no attempt was made to run cultures for longer than seven days, we believe that such cultures can probably be maintained for longer periods of time. One interesting phenomenon noted here was a tendency for the macrophages in the test group to collect in small heaps or mounds consisting of several layers of macrophages. This was not seen in parallel cultures of normal macrophages growing in flasks which were not inoculated with salivary glands. In the normal cultures the macrophages tended to remain in a single layer of cells on the coverslip. In the test group, however, some macrophages apparently left the coverslip (leaving bare areas of glass) and collected in groups of several layers. This produced alternate areas of bare glass and mounds of cells. This began to occur about



1 2 3 4

Plate I. Preparations stained with Giemsa

Fig. 1. Macrophages containing cryptozoites.
Fig. 2. Metacryptozoites in seven day old culture.
Fig. 3. Macrophage containing 18 cryptozoites.
Fig. 4. Ruptured segmenters liberating merozoites.

the fourth day after inoculation of the salivary glands. This phenomenon may have been an attempt on the part of macrophages to collect at points of concentration of bacterial or cellular debris.

Another feature was the loss of synchronism in the development of successive generations of parasites, so that at the end of seven days, one would sometimes see within the same host cell all the stages of development of the parasite, from a newly-developing merozoite to a mature schizont. (Plate II, figures 2 and 3.)

VII. Infection of chicks with pre-erythrocytic stages of P. gallinaceum *grown in vitro*

Now that we were able to maintain cultures of pre-erythrocytic stages of *P. gallinaceum* as long as a week, it was possible to determine whether such parasites grown *in vitro* were capable of producing infection in chicks. Accordingly, scrapings were obtained from cultures 36, 48, 62, 96, and 144 hours respectively after inoculation of the sporozoites into the flask. Such scrapings from some flasks in all of these groups produced infections in chicks. For controls we studied the survival time of the sporozoites *in vitro* in the absence of host cells and found that the sporozoites did not survive longer than 48 hours under the conditions of our experiments. These results are recorded in greater detail in a subsequent paper (Laird, Dubin and Drinnon 1950).

VIII. Morphology of pre-erythrocytic stages of P. gallinaceum *grown in vitro*

In general the appearance of the cryptozoites and metacryptozoites obtained from our cultures was similar to that of the pre-erythrocytic stages developing in the tissues of chickens described by Huff and Coulston (1944), and to that of the exoerythrocytic stages grown in tissue culture by Hawking (1945) and by the senior author (Dubin—). (Plates I and II.) One morphological feature, however, was noted in the cryptozoites which was not seen by the senior author in tissue culture of exoerythrocytic stages developing in cultures from infected chick embryo spleen. This was the presence of one or more sharply-pointed cytoplasmic spurs in the medium-sized schizonts. These spurs were quite distinct and were different from angulations and distortions produced in the periphery of the parasite by the pressure of fat vacuoles in the cytoplasm of the macrophages. These cytoplasmic tails were seen only in the first generation of pre-erythrocytic stages.[7]

IX. Similar experiments with human malarial parasites

Two attempts were made to obtain development of sporozoites of *Plasmodium vivax* in tissue cultures of human tissues, using methods which were successful in the experiments with avian material described above.[8] Both of these experiments failed however. In both experiments the nutrient medium consisted of 20 per cent human

[7] The photomicrographs were made by Charles F. Brock, Jr.

[8] The *Anopheles quadrimaculatus* mosquitoes infected with *P. vivax* were furnished us through the generosity of Dr. Martin D. Young and Dr. Geoffrey M. Jeffery of the Laboratory of Tropical Diseases of the Public Health Service in Columbia, S. C. and Milledgeville, Ga. respectively.

serum in Tyrode's solution. In one experiment, sporozoites of *P. vivax* were inoculated into six day old cultures of human bone marrow obtained from the sternum of a young white male.[9] This patient was afebrile and was recovering from a mastoid operation. He had had no history of malaria. In this experiment there was a good growth of macrophages and various types of bone marrow cells. After inoculation of the salivary glands containing sporozoites of *P. vivax*, the cultures continued to show good growth of cells. No cryptozoites were seen however. Few or no bacteria were seen in the flasks.

In the second experiment, the sporozoites were inoculated into 16 day old cultures made from the liver of a 52 year old white female. The specimen of liver was removed during a cholecystectomy.[10] The cells which grew out from the explant of liver were elongated cells, presumably fibroblasts or Kupffer cells. No parenchymatous liver cells were identified. Six flasks were inoculated with 15 pairs of glands each. Three of the six flasks were destroyed by bacteria. In the other three flasks, there was no interference with cellular growth. No cryptozoites were seen. Further experiments will have to be done along these lines with cultures of human tissues, especially human liver.

It seems that the major problem to be solved at present is how to obtain tissue cultures of identifiable human Kupffer cells and liver parenchymatous cells. The development of human malarial parasites from sporozoites to cryptozoites *in vitro* will have to wait upon the solution of this problem.

SUMMARY

1. The sporozoites of *Plasmodium gallinaceum* developed into cryptozoites *in vitro* following the inoculation of salivary glands of infected *Aedes aegypti* mosquitoes into tissue cultures of normal chicken macrophages.

2. A schedule of antibiotic dosage was worked out which allowed the maintenance of bacteria-free cultures of pre-erythrocytic stages up to 7 days of incubation.

3. Penicillin and streptomycin when used in combination in tissue cultures had a synergistic damaging effect on macrophages and on the exoerythrocytic stages of *P. gallinaceum.*

4. Attempts to obtain development of sporozoites of *P. vivax* into cryptozoites in tissue cultures of human liver and bone marrow were unsuccessful.

[9] The specimen of bone marrow was obtained through the courtesy of Dr. B. D. Hall, Department of Medicine, John Gaston Hospital, Memphis.

[10] The specimen of liver was kindly supplied by Dr. William Tyson, Department of Surgery, John Gaston Hospital, Memphis.

EXPLANATION OF PLATE II

PLATE II. All preparations made from a seven day old culture of pre-erythrocytic stages

Fig. 1. A macrophage containing about 25 very young metacryptozoites. These parasites probably represent the fourth generation of pre-erythrocytic stages in the culture.

Fig. 2. This field contains one segmenter and several very young and intermediate forms. This illustrates the loss of synchronism in the development of the parasites.

Fig. 3. This field also presents pre-erythrocytic stages in various phases of development and again illustrates the loss of synchronism. Also note the presence of several segmenters.

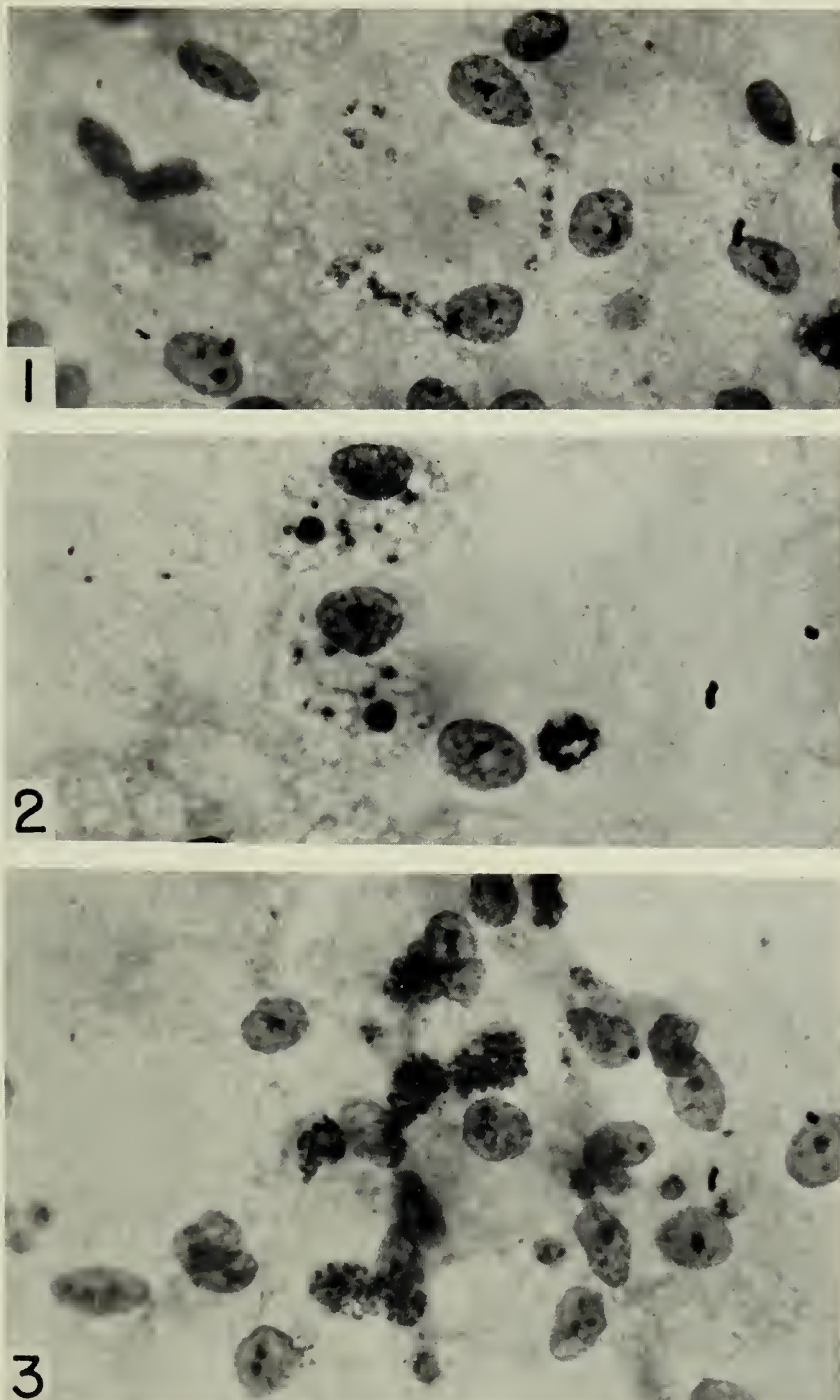


Plate II

RESUMEN

1. Esporozoítos de *Plasmodium gallinaceum* se desarrollaron en criptozoítos, *in vitro*, después de la inoculación de glándulas salivales de mosquitos de *Aedes aegypti* infectados en cultivos de tejidos de macrofagos normales de pollos.

2. Se descubrió una fórmula de dosificación de antibióticos la cual permite el mantenimiento de cultivos de los estadios pre-eritrocíticos libres de bacterias hasta 7 días de incubación.

3. Penicilina y estreptomicina cuando se usan en combinación en cultivos de tejidos tuvieron un efecto sinergético perjudicial contra los macrofagos y estadios exoeritrocíticos del *P. gallinaceum*.

4. Intentos para lograr el desarrollo de esporozoítos de *P. vivax* en criptocitos en cultivos de tejidos de hígado humano y médula de hueso fueron...?

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THE INFECTION OF CHICKS WITH PRE-ERYTHROCYTIC STAGES OF *PLASMODIUM GALLINACEUM* GROWN IN TISSUE CULTURE[1]

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Since the first attempts at tissue culture of avian malarial parasites suspensions of such cultures have been inoculated into the vertebrate host in order to demonstrate the presence of viable parasites. Huff and Bloom (1935), Gavrilov, Bobkoff and Laurencin (1938), Hegner and Wolfson (1939), Hawking (1945) and others have used this method of demonstrating viable parasites in tissue cultures. These previous experiments were all done with cultures of post-erythrocytic tissue parasites of avian malaria. Our report, on the other hand, presents the results of inoculation of chicks with pre-erythrocytic parasites developing from sporozoites in tissue culture.

MATERIALS AND METHODS

Cultures of macrophages were prepared and inoculated with sporozoites as described elsewhere (Dubin, Laird and Drinnon, 1950). An average of five pairs of infected glands were inoculated into each flask. After suitable incubation, suspensions of the cultures were prepared by scraping the bottom of the flask with a platinum wire and suspending the scrapings in the medium already in the flask. The suspension was then drawn into a one cubic centimeter syringe and inoculated intravenously into week-old chicks.

For controls, tests were set up to determine the survival period of sporozoites under similar conditions of temperature, pH and nutrient medium, but in the absence of macrophages. In these experiments a series of flasks containing the liquid medium, but devoid of cells, were inoculated with equal numbers of infected salivary glands and incubated for various periods of time. The suspensions obtained from these flasks were inoculated as above.

Blood smears were made daily from the inoculated birds for a period of 21 days following inoculation. Each smear was examined by two persons and at least 10,000 red blood cells were reviewed by each examiner before the smear was considered negative.

RESULTS

Table 1 summarizes the results of these experiments. The infection in the chicks showing erythrocytic parasites followed the life cycle pattern designated as "A" by Haas and co-workers (1948). This is the normal life cycle pattern of *P. gallinaceum*

[1] A contract with the Office of Naval Research, Microbiology Branch, Number N8onr-75700, and cooperation of the Tennessee Valley Authority through financial assistance provided under contractual agreement have made this work possible.

in chicks established by passing the parasites from chick to chick through mosquitoes. The prepatent period of chicks infected in the present experiments lasted from 7 to 13 days. The erythrocytic parasites in these birds appeared normal. An abundance of gametocytes were present and mosquitoes fed on these chicks became heavily infected. One striking difference from the usual pattern, however, was noted in the birds infected with pre-erythrocytic parasites grown in tissue culture. While chicks infected with sporozoites usually die in from 14 to 20 dyas post-inoculation, the majority of the chicks infected with pre-erythrocytic parasites survived, in spite of the fact that many showed parasitization of over 50 per cent of the red blood cells. This difference in survival might be accounted for by some possible alteration of the parasite *in vitro*.

TABLE 1

Comparison of results obtained by inoculating chicks with surviving sporozoites and those obtained by inoculating chicks with pre-erythrocytic parasites grown in vitro

		AGE OF CULTURE						
		1 hr.	20 hrs.	36 hrs.	48 hrs.	62 hrs.	96 hrs.	144 hrs
Inoculation of surviving sporozoites	No. of chicks positive	2	1	0	1	0	0	0
	No. of chicks inoculated	2	3	4	3	3	5	4
Inoculation of preerythrocytic parasites grown *in vitro*	No. of chicks positive	—	—	1	4	7	4	1
	No. of chicks inoculated	—	—	4	4	8	6	4

The results summarized in table 1 indicate that sporozoites can survive up to 48 hours in the culture medium and produce infection when inoculated into susceptible animals. Although there was microscopic evidence of growth of pre-erythrocyic forms at a much earlier period than this, it would still be possible for infections in chicks to arise from surviving sporozoites as late as 48 hours after their introduction into the culture flasks. Cultures examined at 48 hours or less showed occasional poorly stained and abnormal looking sporozoites while those examined after longer periods of incubation gave no microscopic evidence of sporozoites, even degenerate ones. In view of the biological and microscopic evidence it seems clear that the infections in chicks inoculated with scrapings from flasks 62 hours or more after sporozoites had been introduced were the results of pre-erythrocytic parasites growing *in vitro*.

A series of experiments was set up in order to demonstrate the pattern of infection produced by pre-erythrocytic parasites grown in tissue culture. Sporozoites were inoculated into cultures of macrophages and after three days suspensions of these cultures were inoculated into chicks. When erythrocytic parasites (including gametocytes) appeared in the chicks, *Aedes aegypti* mosquitoes were fed on the

birds. One hundred per cent of these mosquitoes became infected. Sporozoites from some of these mosquitoes were inoculated into cultures of macrophages and sporozoites from the remainder were inoculated into chicks. All the chicks became infected showing pattern "A". The macrophage cultures showed microscopic evidence of development of cryptozoites. This series of experiments is illustrated in figure 1.

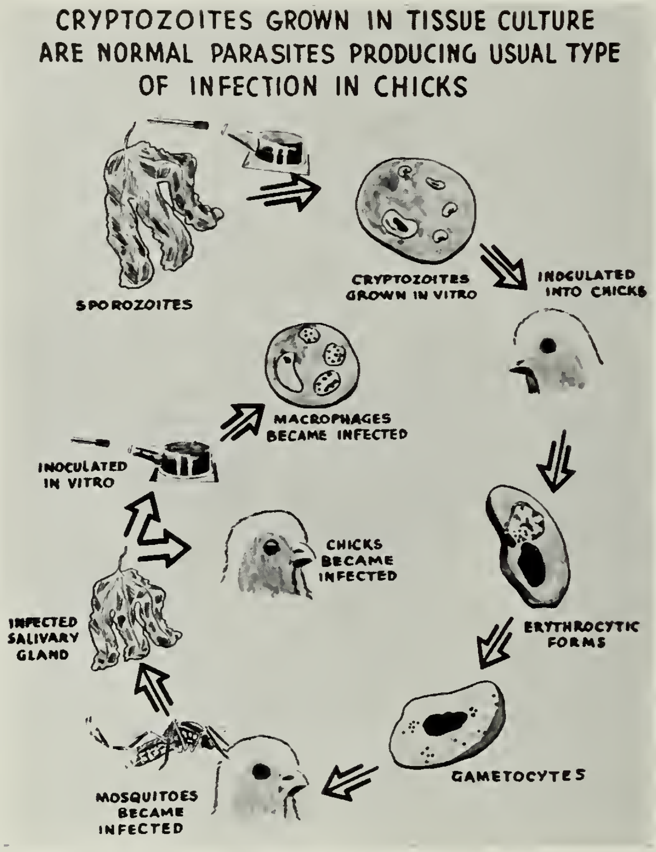

Fig. 1. Diagramatic Illustration of Cycle of Parasite Development

DISCUSSION

The inability of other workers to grow pre-erythrocytic parasites in tissue culture was probably due to difficulties in techniques. Porter (1948) gave indirect evidence that either sporozoites survived in the cultures or that there was some growth of pre-erythrocytic forms. In one experiment he distributed a suspension of sporozoites of *P. gallinaceum* into 11 macrophage cultures. Forty-eight hours later he inoculated these cultures into chicks. One of the 11 chicks exhibited parasites 15 days after inoculation. In a later experiment two cultures incubated 48 hours produced infections when inoculated into chicks. Sporozoites were introduced into these latter cultures at the same time that the leucocyte suspension was added.

Hawking was unable to grow cryptozoites in tissue culture by inoculating sporozoites directly into the cultures. However, when he inoculated chicks with large numbers of infected glands and explanted the spleens from these chicks, one hour later he was able to demonstrate growth of the parasites in the tissue cultures. In view of the fact that Huff and Coulston (1944) showed the presence of fairly normal looking sporzoites in tissue cells one hour after inoculation of the animals, Hawking may have explanted with his spleens fairly large numbers of intracellular sporozoites which under favorable conditions grew into cryptozoites. In the present experiments the survival of sporozoites dropped rapidly after inoculation into the flasks although one flask out of three showed viable sporozoites at the end of 48 hours. However, in the flasks containing macrophages, infective parasites were present in four out of four flasks incubated for the same period. It seems reasonably certain that the greater infectivity of these latter cultures was due to the cryptozoites growing *in vitro* rather than to surviving sporozoites.

SUMMARY

Data has been presented which demonstrate that the pre-erythrocytic forms of *P. gallinaceum* developing from sporozoites in tissue culture are infective for chicks and produce a normal life cycle pattern.

Sporozoites of *P. gallinaceum* can remain viable for 48 hours in dilute chicken serum kept at 37° C. and pH of 7.4.

RESUMEN

Se presentan datos que demuestran que las formas pre-eritrocíticas de *P. gallinaceum* provenientes de esporozoítos de cultivos en tejidos son infectivos a los pollos y producen un ciclo de vida normal.

Esporzoítos de *P. gallinaceum* permanecen vivos por 48 horas en suero de pollo diluído mantenido a 37° C. y a un pH de 7.4.

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SURVIVAL AND GROWTH OF FOUR SPECIES OF AVIAN *PLASMODIA* ON THE HARVARD CULTURE MEDIUM[1]

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For many years avian malaria was almost the only practical tool for the laboratory study of malaria. Although this is no longer true, since simian malaria is being used in a number of laboratories, and *Plasmodium berghei* of the rat will, it is hoped, soon become widely available, malariologists nevertheless know that we shall never have a really complete understanding of malaria in man until we have a much greater knowledge of what may be called "comparative malariology" than we do now. It is therefore very important that we continue an intensive study of the malarias of the lower animals.

Of all the procedures available for such work not any are of more potential value than the cultivation techniques, imperfect though they still are. By their use we may hope to learn not only much about plasmodial physiology and biochemistry, and about the mechanism of drug action, but even something about the nature and possibly the origin of the host-parasite relation.

The evolution of pathogenic from non-pathogenic species is today being regarded as perhaps in part the consequence of the loss of certain enzymes, or enzyme systems, with the result that when this happens the metabolism of the parasite no longer parallels that of the host and toxic by-products accumulate. Trypanosomes have been divided into two groups on this basis, the one relatively harmless to the host and the other often lethal. If our knowledge of the different species of plasmodia were complete enough, we might also perceive interesting relationships of a parallel kind among them.

The culture tube may be regarded as a kind of artificial host, in which the number of variable factors may be held to a minimum. The avian plasmodia offer especially favorable material for studies of this sort. From such research we may hope to learn much of the biochemical and physiological differences which separate species, for at least 15 such species are known and it is probable that their avian hosts are much more closely related biologically than are most of the mammals which harbor plasmodia. Thus it is at least likely that the avian plasmodia are more similar among themselves than are the corresponding species in mammals.

So far few species of avian malaria parasites have been successfully cultivated. By the use of the tissue culture technique, exoerythrocytic stages of *Plasmodium gallinaceum*, *relictum*, and *lophurae* have been grown (Hawking, 1945; '46; Tonkin and Hawking, 1947; Dubin, Laird and Drinnon, 1949). An earlier and less successful attempt to culture *Plasmodium cathemerium* by this means was that of Hegner and Wolfson (1939). But although Trager (1941, 1943, 1947, 1949) has been able to achieve some multiplication of the erythrocytic stages of *Plasmodium lophurae* in cultures, little or no work of this kind has yet been reported on the other species.

[1] Aided in part by a grant-in-aid from the National Institute of Health.

MATERIALS AND METHODS

This study has included attempts to cultivate four species of avian plasmodia: *Plasmodium gallinaceum*, *lophurae*, *cathemerium*, and *relictum*. We have used the medium and process developed by Ball, Geiman, McKee, and their colleagues (Ball *et al.*, 1945; Anfinsen *et al.*, 1946; Geiman *et al.*, 1946) for *Plasmodium knowlesi* at Harvard, for the most part without essential modification. Such modifications as have been made are indicated in Table 2, part B. Seitz filters were used, except for the Vitamin C fraction, which was passed through a Corning ultra-fine fritted glass filter.

Of the two types of culture tube devised by them, the rocker perfusion was proved more useful and has been employed in most of our experiments. This is partly because it is better adapted to the use of small quantities of blood.

The strains of *Plasmodium gallinaceum* and *lophurae* are those which have been used in other laboratories in this country and abroad. Our strain of *P. cathemerium* (officially designated by the Committee on Terminology of Strains of Avian Malaria as "3E") was originally isolated by the senior author from a chipping sparrow caught at the Patuxent Wild Life Refuge in Patuxent, Maryland, in 1944. We have used a morning-segmenting strain ("1P") of *Plasmodium relictum* originally isolated by Dr. G. Robert Coatney (1937) from a pigeon, and supplied to us through the courtesy of the Army Medical Department Research and Graduate School. We have noted no significant differences between this strain of *P. relictum* and that designated by Huff "var. *matutinum*" except that the latter produces a larger number of merozoites per segmenter (a mean of 14,[2] as compared to 11).

These strains of malaria have been carried by us in chickens, ducks, and canaries, according to convenience and host susceptibility. Infected blood for cultures has been obtained from chickens in the case of *Plasmodium gallinaceum* and *lophurae*, and from ducklings not more than two weeks old for the other two species.

The progress of cultures was gauged at fairly regular intervals of from 8 to 12 hours by examination of J.S.B.-stained smears, and (as a test of viability) by inoculation into fresh birds. Infections in the donor birds were also carefully followed, so that development in the cultures could be checked with that occurring simultaneously *in vivo*. This involved studies of the normal course of infections of this strain of *Plasmodium relictum* in the duck, as described below.

Table 1 summarizes the experiments done. Since it was early found that, of all the species used for cultivation, *Plasmodium relictum* gave the most consistently good results, the greater amount of work was done on this species.

RESULTS

Since any evaluation of the success of a culture technique involves a comparison of the behavior of the parasites in the host and in the test tube, the infection of the duckling with *Plasmodium relictum* will be described first. That the matinal strain

[2] It is probable that a number of differing strains exist, even of this variety. Wolfson (1937), for example, states the range of merozoites per segmenter to be 16 to 24 in her strain. However such figures are likely to vary somewhat, depending on the species of host and even the stage of the infection.

will infect young ducks has been known since the work of Wolfson (1939) and Manwell and Hatheway (1943), but we have found that the "1P" strain in the duck behaves rather differently from the strains used in the earlier work.

Ducklings inoculated intravenously with parasites when two or three days old usually develop infections in another three or four days, unless the inoculum is de-

TABLE 1

*Summary of Cultivation Experiments**

SPECIES OF AVIAN MALARIA	NUMBER OF EXPERIMENTS	TYPE OF NUMBER OF CULTURE TUBES†		MAXIMUM DURATION
		R.D.	R.P.	
				hours
Plasmodium relictum				
a) Harvard Medium	8	3	15	48
b) Medium or blood fraction modified	11	—	21	48
Plasmodium gallinaceum	8	5	16	63
Plasmodium cathemerium	3	—	15	24
Plasmodium lophurae	4	—	6	63

* All experiments were done using the Harvard Medium, except as noted for *Plasmodium relictum.*
† "R.D." = rocker dilution tube.
"R.P." = rocker perfusion tube.

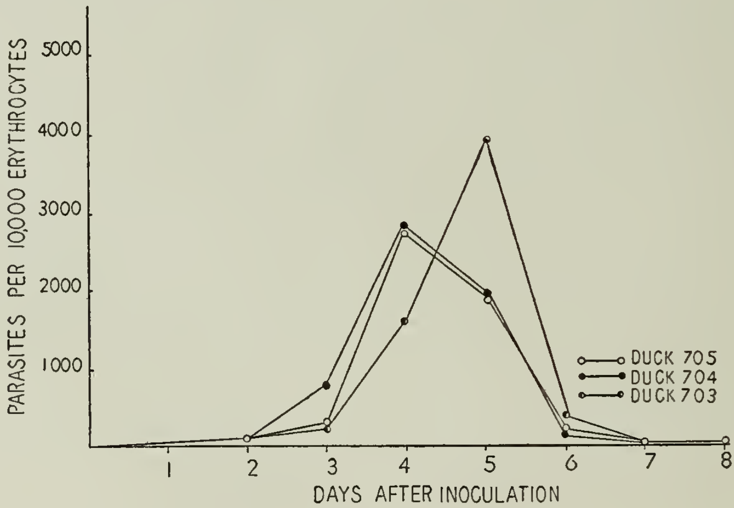

FIG. 1. *Plasmodium relictum* ("1P") in young ducks. Note the rapid rise and fall of the parasitemia, and the sharply marked crisis.

rived from a chronic case. In the latter event the prepatent period may amount to a week or even more. The acute stage is remarkable for the rapidity of the rise in parasitemia, and its equally rapid fall after a sharply marked crisis. (Figure 1.) Although the parasite count may reach 3,000 or 4,000 per 10,000 red cells within two or three days after the parasites first appear, it often drops to less than a tenth of this figure in 24 hours, and within another day it may be close to zero. Yet the ducks

retain a chronic infection for weeks, and perhaps indefinitely. In this respect the "1P" strain of *P. relictum* is similar to Wolfson's but quite different from the one used by the senior author in earlier work. The "1P" strain also develops more quickly after infection by blood inoculation, and produces a considerably higher parasitemia. But, paradoxically, it is also much less pathogenic. We have never had a death in several hundred infections, as compared to a mortality of about 14 per cent in ducks in the strain reported on by the senior author in 1943. Relapses are also very seldom seen.

Although the schizonts often appear elongate (in contrast to their round or rather irregular shape in the canary), the gametocytes in the duck are usually round. Wolfson (1939) observed that nearly 80 per cent of the gametocytes of her matinal strain of *P. relictum* appeared elongate in the duck.

A further peculiarity of the parasites of the "1P" strain in the duck is their strong preference for reticulocytes. Not infrequently more than 90 per cent will be found in such cells. It is true that the proportion of reticulocytes to mature red cells is often rather high in the course of a malarial infection, but the proportion of parasites found in cells of the former type is double or triple what might be expected from chance alone. This is probably a limiting factor of consequence in cultivation.

As in other matinal strains of *Plasmodium relictum*, periodicity and synchronicity of this one are sharply marked, but less so in the duck than in the canary. Segmentation in the duck takes place between 6 and 10 A.M., and by noon 90 per cent o the parasites are trophozoites. (cf. Coatney, 1940; Redmond and Prather, 1943 for a discussion of periodicity phenomena of this strain in the pigeon and canary.)

Success in cultivation was measured primarily by what we have called the "Growth Index", or the ratio of parasite population at the end of a period of cultivation to that at the beginning. This was based on calculations of the number of parasites per 10,000 red cells, enough parasites being always counted to keep the probable error within 10 per cent.

The average Growth Index after 20 to 24 hours of cultivation of *Plasmodium relictum* was found to be 2.12 $\pm$ 0.09 when the unmodified Harvard medium and technique was used, although we found, as others have using other species of plasmodia, a good deal of variation in the results of different experiments, even when conditions seemed quite uniform.

Figure 2 shows the growth indices obtained in a number of different cultivation experiments involving this strain of *P. relictum*. In most cases the period of cultivation, the results of which were graphed, was 24 hours, but in several cases parasite multiplication after that is indicated by dotted bars superimposed on those shown in solid lines.

When culture tubes are run for longer than 24 hours, the rate of increase rapidly levels off and there may even be an apparent decrease in parasite population after 36 hours. At the same time the mean number of merozoites per segmenter diminishes. In the early culture period (8 to 12 hours) it amounts to 12.42 $\pm$ 0.11, then becomes progressively less. After 24 hours it is 8.98 $\pm$ 0.10, and after 36 it is only 8.77 $\pm$ 0.15. These figures may be compared to 10.87 $\pm$ 0.13 per segmenter, the mean for the duck.

Subcultures were not attempted, but subinoculation shows that parasites remain viable for at least 48 hours.* Ducklings inoculated from such cultures developed normal infections after prepatent periods of the usual length, indicating that a large proportion of the contained parasites were quite able to reproduce normally after transfer to their accustomed environment. The results of our cultivation experiments with *P. relictum* are summarized in Table 2.

There is reason to think that segmentation is at first somewhat accelerated in culture, but the high degree of synchronicity and sharp periodicity exhibited *in vivo* seem to be lost.

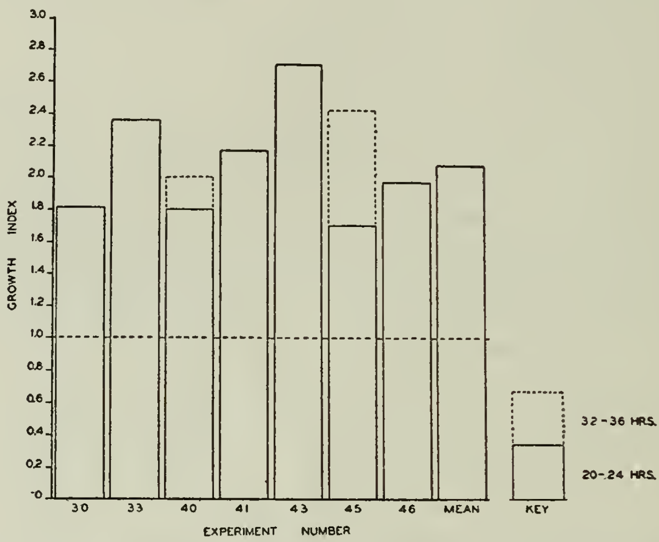

FIG. 2. Growth Indices of *Plasmodium relictum* ("1P") cultivated in Harvard Medium. Bars shown in heavy lines represent growth periods of 24 hours; dotted extensions show 36 hour Growth Indices for comparison, as observed in two experiments. Note drop in multiplication rate after the first 24 hours.

The appearance of parasites in stained preparations is a fairly good index of their condition. We have found that the erythrocytes from cultures are more easily destroyed in the making of smears, and hence it seems probable that prolonged exposure to the medium makes them more fragile. On the whole, the parasites appear to be less affected. Figures 1 to 8 (Plate 1) show the morphology of parasites after periods of culture of from 8 to 40 hours, together with similar pictures of parasitized blood obtained *in vivo* for comparison. Gametocytes seem to withstand culture longer than asexual forms, but this statement is based on their appearance rather than on exflagellation tests.

Because the original host of this strain was the pigeon, and because the strain also goes well in the canary, pigeon plasma or canary serum was substituted for duck plasma in the whole blood fraction of some of the cultures. It is of considerable interest, especially as it is known that the plasma of some species of birds varies

*Later work has shown viability to at least 65 hours.

TABLE 2

Cultivation of Plasmodium relictum

A. Harvard Medium

EXPERIMENT NUMBER	TYPE AND NUMBER OF TUBES†		GROWTH INDEX (HOURS)*				PROVED VIABLE AFTER
	R.D.	R.P.	8–12	20–24	32–36	44–48	
							hrs.
30	—	1	—	1.86	—	—	—
33	—	1	1.48	2.36	—	2.16	—
40	—	2	1.18	1.80	2.00	1.06	—
41	—	3	1.47	2.18	1.30	—	36
43	—	3	1.43	2.71	2.76	1.49	—
45	—	1	1.79	1.70	2.42	1.30	48
46	3	2	1.25	1.97	—	1.51	48
47	—	2	—	1.97	—	—	—

B. Medium or Blood Fraction Modified

NATURE OF MODIFICATION	EXPERIMENT NUMBER	TUBES	GROWTH INDEX (20–24 HOURS)		RATIO EXP./CON.†
			Exp.†	Con.†	
a. Whole blood fraction					
Whole blood, normal duck plasma, 1/1 ratio	40	2	2.28	1.80	1.26
	41	3	2.31	2.18	1.06
Whole blood, normal chick plasma, 1/1 ratio	40	2	2.05	1.80	1.14
Duck blood with plasma removed, and equal amount of canary serum substituted	43	2	4.34	2.71	1.60
	45	2	1.12	1.70	0.65
	46	1	1.76	1.24	1.42
	47	2	3.03	1.97	1.58
Duck blood with plasma removed, and equal amount of pigeon plasma substituted	45	2	1.33	1.70	0.78
	46	1	2.38	1.24	1.92
b. Changes in Medium					
Purines and pyrimidines increased 10 fold	46	2	2.22	1.24	1.79
Glucose increased 10 fold	46	2	1.53	1.24	1.23

* Whenever more than one tube was run in a single experiment, the Growth Index is given as the mean of all the tubes. In interpreting these values, it is essential to remember that when the period of cultivation is less than 24 hours it may include only part of the time required for segmentation (or possibly none of it), in which case the Growth Index will be necessarily low.

† Abbreviations: "R.D." = rocker dilution tube.
"R.P." = rocker perfusion tube.
"Exp." = experimental cultures.
"Con." = control cultures.

in the character and amounts of contained proteins (and no doubt of other substances), that the growth index in two of these experiments considerably exceeded the best record made otherwise. The maximum obtained was 4.34, using canary

serum. The corresponding figure for the control tubes in which duck plasma was used was 2.71. In another experiment in which pigeon plasma was used, the growth index (2.38) was less striking, but this was also double that of the duck plasma control. The results of several other experiments of this kind were less favorable however, and it is essential that similar studies be continued.

TABLE 3

Cultivation of Plasmodium gallinaceum

(Harvard Medium)

EXPERIMENT NO.	TYPE AND NUMBER OF TYBES†		GROWTH INDEX (HOURS)*						PROVED VIABLE AFTER
	R.D.	R.P.	8–12	16–20	20–24	32–36	44–48	63	
									hrs.
18	1		—	0.67	—	—	—	—	16
19		1	—	1.33	—	0.44	—	—	38
24	2		—	0.98	—	—	—	—	—
25	2		—	0.74	0.78	—	—	0.14	63
26	1	3	—	—	1.08	—	0.26	—	48
31		3	1.17	0.52	—	—	—	—	—
32	1	2	1.19	1.56	—	1.07	—	—	—
36		5	1.30	1.76	1.60	—	—	—	—

* Wherever more than one tube was used in a single experiment, the Growth Index is given as the mean of all the tubes.

† "R.D." = rocker dilution tube.
"R.P." = rocker perfusion tube.

EXPLANATION OF PLATE 1

The right half of each illustration shows parasites and red cells from a J.S.B.-stained preparation made directly from a *Plasmodium relictum*-infected duck, and the left half is from a similar preparation of cultured blood from the same duck. Both duck and culture were smeared at as nearly the same time as possible. Magnification: 1200×.

Figures 1 and 2: Parasites at midnight, after 8 hours of culture (Figure 1). Both parasites and red cells still look quite normal, and the stage of parasite development is about the same *in vivo* and *in vitro.*

Figures 3 and 4: Parasites at 8 A.M., after 16 hours of culture (Figure 3). Segmentation is taking place. Note the two young parasites at each end of the reticulocyte at the bottom of Figure 4; multiple infection and invasion of reticulocytes are both especially frequent in ducks infected with this strain of *P. relictum.*

Figures 5 and 6: Parasites at 4 P.M., after 24 hours in culture (Figure 5). Some segmentation is still taking place in the culture, whereas in the duck the asexual forms are all trophozoites or even young schizonts. The plasmodia still appear quite normal, although there is a suggestion of beginning degeneration in the cytoplasm and nuclei of some of the erythrocytes.

Figures 7 and 8: Parasites at 8 A.M., after 40 hours of culture (Figure 7). In the duck young trophozoites predominate, but the parasites in the culture are in various stages of development. Shown in Figure 7 is a group of merozoites, abnormal both in appearance and in number (8 compared to the usual figure of 11). Compare with the group of merozoites in Figures 3 and 5 after shorter periods of culture.

* Microphotographs by Miss Stella Zimmer, Department of Photography, Syracuse University College of Medicine.

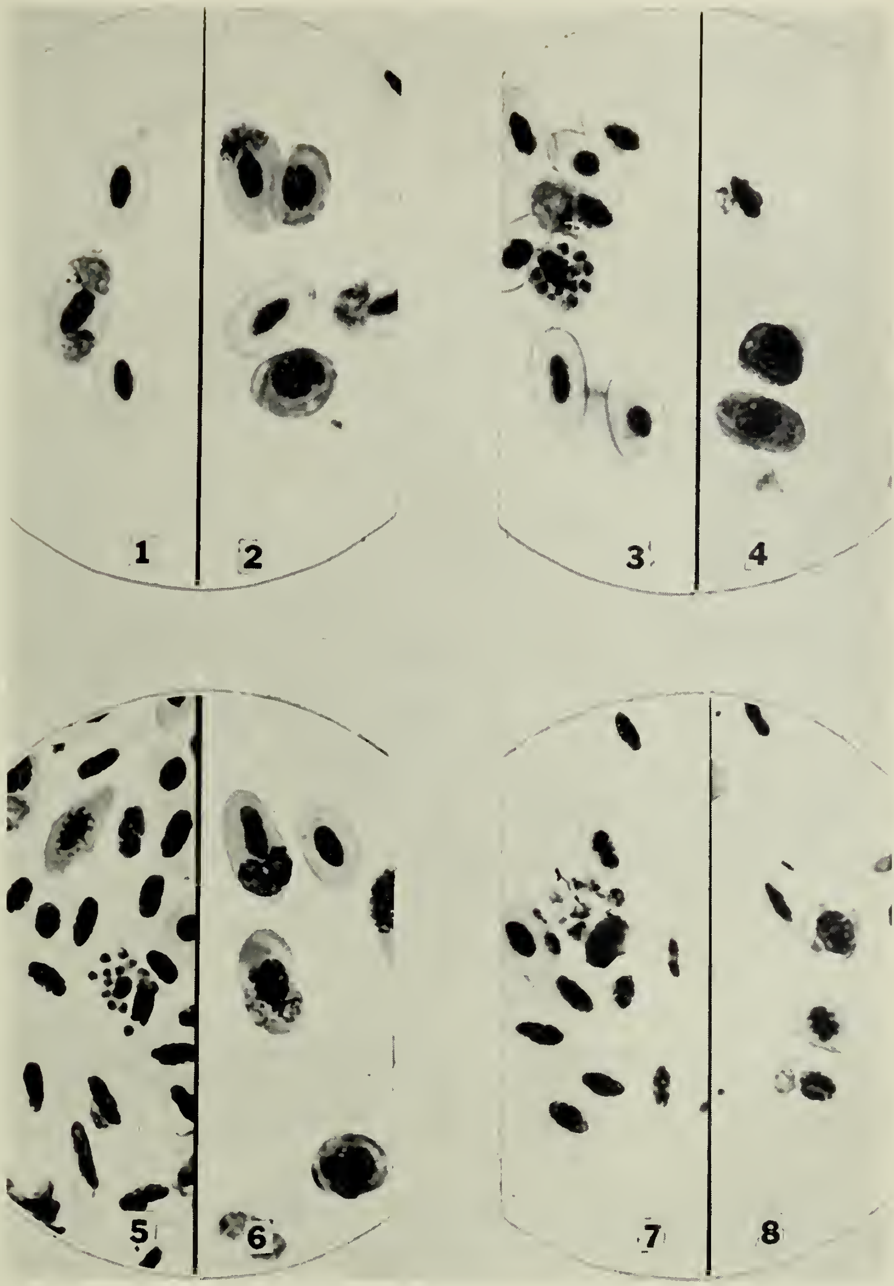

Plate 1*

Other modifications in the standard Harvard medium, or in the blood mixture, included diluting the blood with additional duck or chicken plasma, and 10-fold increases in purines and pyrimidines, and (in another experiment) a similar increase in glucose. In none of these cases did the changes alter the performance of the medium significantly.

The results of attempts to cultivate *Plasmodium gallinaceum* are summarized in Table 3. In no case was any real success obtained. Some multiplication occurred, but the best record in 24 hours gave a growth index of only 1.60. However viability tests showed that the parasites in some cultures lived at least 63 hours, although not all the birds so inoculated developed infections.

TABLE 4

Cultivation of Plasmodium cathemerium*

(Harvard Medium)

EXPERIMENT NO.	TYPE AND NUMBER OF TUBES‡		GROWTH INDEX (HOURS)†		
	R.D.	R.P.	8–12	16–20	20–24
30	1	1	1.14	1.14	1.12
33	—	6	1.26	1.13	0.93
34	—	7	1.36	1.24	1.13

TABLE 5

Cultivation of Plasmodium lophurae

(Harvard Medium)

EXPERIMENT NO.	TYPE AND NUMBER OF TUBES‡		GROWTH INDEX (HOURS)†				PROVED VIABLE AFTER
	R.D.	R.P.	16–20	20–24	32–36	63	
							hrs.
18	1	—	1.00	—	—	—	—
23	2	—	1.41	—	0.36	—	37
25	1	—	1.16	0.63	—	0.18	63
27	1	1	1.10	0.63	—	—	—

* No viability tests were done on this series.

† Whenever more than one tube was run in a single experiment, the Growth Index is given as the mean of all the tubes.

‡ "R.D." = rocker dilution tube.
"R.P." = rocker perfusion tube.

Trial of the medium on *Plasmodium cathemerium* was even less successful. The mean 24-hour growth index, based on three experiments and 15 tubes, was only 1.02, or less than half that obtained with *Plasmodium relictum*. Part of the failure of *P. gallinaceum* to show much multiplication in the first 24 hour period may have been due to the time of day when cultures were started, since this species has a 36 hour cycle, but both *P. cathemerium* and the "1P" strain of *P. relictum* have a 24 hour cycle. Thus it is clear that either there was no liberation of merozoites in the cultures or a very heavy parasite mortality. For this reason no viability tests were done. Experimental results for this species are given in Table 4.

A few attempts were also made to use the medium for the culture of *P. lophurae.* The results are summarized in Table 5. In terms of the growth index obtained after 24 hours of culture these experiments were the most disappointing of all, since it averaged only 0.63 ± 0.12, and the parasite population dropped steadily until after 63 hours it was less than 20 per cent of what it was at the beginning. Nevertheless, in some cultures at least, there were still virulent parasites after this length of time, as proved by the successful infection of young chicks. It is also to be remembered that this species has a longer cycle than the other three (48 hours according to Terzian, 1941; 36 to 48 according to Trager, 1947). It is true that there was some multiplication in most of the tubes in the first 16 to 20 hours, but the parasite mortality thereafter was progressive and rapid. Figure 3 shows composite curves for all four species for the entire duration of the experiments.

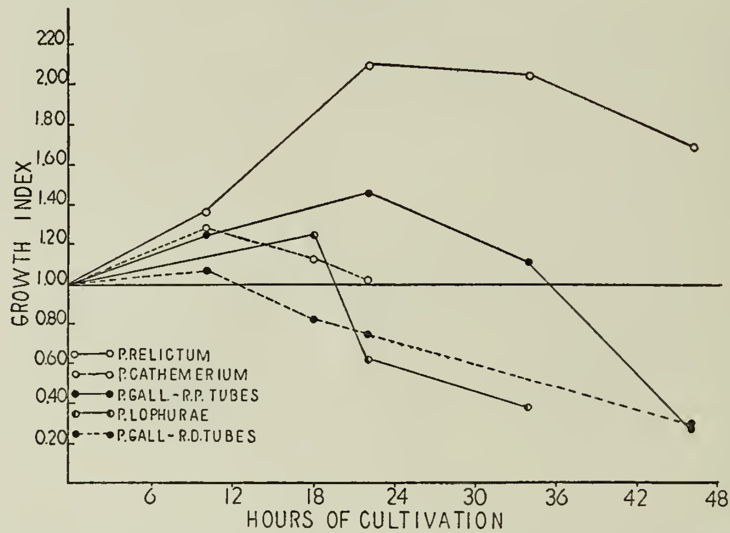

FIG. 3. Growth Indices for the four species compared for the total period of cultivation.

DISCUSSION

It is apparent that the Harvard medium and culture technique are not well suited to the four species of avian plasmodia on which we have tested it, although it gives better results on *Plasmodium relictum* than on the other three. The average growth index after 24 hours of culture, 2.12, represents a smaller rate of increase than that usually observed in the host during a similar period while the infection is rising, although both *in vitro* and *in vivo* there is a good deal of variation. The maximum rate of increase in 24 hours, 4.34, seen in a culture incorporating canary serum, comes much closer to the normal *in vivo* behavior of the parasites. However a really good cultivation process should give a growth index closely approximating the mean number of merozoites per segmenter released every 24 hours (in strains such as this one, in which the asexual cycle is 24 hours long), since in the culture tube there is no immune mechanism to hold the parasites in check. It should also be sufficiently

standardized to give consistent and uniform results. A study of the literature reveals that as far as the culture of the erythrocytic stages is concerned only the first of these ideals has been very closely approached for any species of plasmodium as yet.

The medium developed by Ball and his co-workers was the result of an attempt to provide environmental conditions for the erythrocytes as nearly similar as possible to those existing in the mammalian host, and thus to continue outside the body the optimum growth conditions for the plasmodia. This end was quite successfully achieved for *Plasmodium knowlesi*, since "In general, an average of about four-fold multiplication was obtained in control experiments with the parasite count increasing as much as six- to ten-fold in many of the cultures. . . . Indeed, cultures often exhibit a greater multiplication in one asexual cycle than that observed *in vivo* during the same period." (Anfinsen *et al.*, 1946, p. 611). Yet, even in their work, there was a marked variation in the results achieved in different experiments.

Aside from these studies at Harvard, the most successful attempts to cultivate the erythrocytic stages of the malaria plasmodia have been those of Trager at the Rockefeller Institute. His methods have, on the whole, been similar to those developed by the Harvard group although he has been at pains to point out that the ideal cultivation method will be one which duplicates the intra-cellular environment of the parasite, rather than one which simply permits the survival of the red cells with their contained plasmodia for a time. The species of parasite used by him was *Plasmodium lophurae*, and the host in his earlier work was the chick. More recently he has used the duck, and with it has achieved considerable success. Our less successful results may have been due to our use of the chick.

Although Hawking's greatest success was gained with his tissue culture technique of culturing the exoerythrocytic stages of *Plasmodium gallinaceum*, he also undertook to repeat the work of Trager, using however this same species rather than *P. lophurae*. Although the parasites survived for several days, and even multiplied somewhat in occasional cultures, he was finally forced to conclude "that no substantial multiplication of the parasites could be achieved." (Hawking, 1945).

Very recently, Trager (1949) has reported fairly normal growth and multiplication of *Plasmodium lophurae* over a 24 hour period on a cell-free, semi-solid medium with a base of gelatin or agar. Good growth for a longer period and successful subcultures however have not yet been obtained with this technique. The only previous work of this sort seems to have been that of Hewitt (1938). He used inspissated whole eggs, to which serum from various animals (the most successful was rabbit serum) and saline containing 0.5 per cent dextrose had been added. Each culture tube was then inoculated with a drop of blood, heavily parasitized with *Plasmodium cathemerium*. Development was obtained, but no merozoites were actually liberated from host cells.

As far as *Plasmodium relictum* is concerned, no previous attempts to cultivate the erythrocytic stages have been reported except the early ones of Manwell and Hewitt (1937). In this work sealed capillary tubes were used, in which heparinized parasitized blood had previously been placed. Addition of glucose did not seem to be essential. Survival of the parasites, as evidenced by their ability to infect fresh canaries, was proved after incubation at 25°C. for as long as five days, but how

much growth and multiplication occurred in the cultures was uncertain. The strain of *Plasmodium relictum* (*praecox*) used in these experiments was not a matinal strain.

In this connection, the attempts of Gordon H. Ball (1947; '48) to cultivate the mosquito phase of *Plasmodium relictum* also deserve mention. No development of the oocysts could be certainly demonstrated, and no proof of viability was had, but some of the parasites retained a quite normal appearance in culture for as long as 20 days.

Another set of experiments in this field which should also receive notice was done by Coulston (1940; 1941). Unfortunately these have never been reported in detail. In his first work, he introduced semi-permeable capsules containing the parasites (*Plasmodium circumflexum*) into the bodies of canaries. If the supply of fresh erythrocytes in the capsules was maintained, the parasites would develop and invade new host cells, and survival for at least as long as 17 days could be demonstrated. Later, he tried to cultivate the same species on a medium not unlike that since devised by Ball, Geiman, *et al.*, and by Trager. The parasites "grew for at least one asexual generation . . . possibly three or four" and infections in canaries could be produced with them even after 13 days of cultivation. If fresh erythrocytes were added at intervals, invasion of new host cells "was easily demonstrated."

Thus it appears that so far no one has been as successful in cultivating the erythrocytic stages of any species of avian malaria as have the Harvard workers in growing *Plasmodium knowlesi*. Nevertheless it seems likely that the media and techniques devised by the Harvard group and by Trager will not require radical modification to adapt them to the culture of other plasmodia, although it may well happen that each species of malaria will prove a little different from any other in its growth requirements.

Quite possibly the difficulties which have so far been encountered are more due to differences in the biochemical requirements of avian erythrocytes, because of their nucleated condition, than to physiological differences separating mammalian and avian plasmodia.

It seems likely that another factor of importance may be the choice of host species. Not only does the differing character of the infection produced in different species of hosts by a given species of plasmodium suggest that these hosts furnish significantly different environments for the parasite, but it is known from the limited amount of biochemical work already done on the blood of certain bird species that the plasma of each is more or less unique in composition. Fortunately for the prospects of future success in work of this sort, the relative lack of host specificity in many of the species of avian plasmodia suggests either that such chemical differences in the plasma of birds are of rather minor degree, or that the plasmodia themselves have somewhat elastic growth requirements.

SUMMARY AND CONCLUSIONS

The culture medium devised by Ball and co-workers for *Plasmodium knowlesi* has been tried on four species of avian plasmodia: *Plasmodium relictum* ("1P" strain), *cathemerium*, *gallinaceum*, and *lophurae*. Success in cultivation has been evaluated in terms of parasite multiplication, appearance in stained preparations, and ability

to infect fresh birds. Young chicks and White Pekin ducklings have been used as hosts.

1. Of the four species, *Plasmodium relictum* has been found to grow best, an average rate of multiplication over a 24 hour period of 2.12 having been achieved. This is however less than the rate of increase commonly observed *in vivo*.

2. Of a number of modifications in the medium or the blood mixture containing the parasites, the only one which appeared to have promise was the substitution of canary serum for duck plasma in cultures of *Plasmodium relictum*.

3. Growth and sporulation of this species in culture appear to be initially stimulated somewhat, and there is a moderate increase in the average number of merozoites per segmenter. This drops off as the age of the culture increases, until after about 36 hours there is an actual drop in parasite population. The rather sharp synchronicity and periodicity in the asexual cycle of this strain are lost in cultures.

4. Parasites may remain viable in cultures for periods at least as long as 63 hours, even when little or no multiplication has taken place. This was shown to be true even of *Plasmodium lophurae*, a species for which the medium seemed especially ill-adapted. Viability of parasites in 65 hour cultures of *P. relictum* was also demonstrated. Staining characteristics begin to change before this however, suggesting that many of the plasmodia are abnormal or degenerate.

5. The behavior of the "1P" strain in the duckling is described, and certain differences between this and other matinal strains are pointed out.

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THE INFECTION OF ANOPHELINE MOSQUITOES BY NATIVE AVIAN MALARIA

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It has been generally accepted for many years that there is a fairly strict host-parasite specificity between mosquitoes and malaria. Anopheline mosquitoes have been considered as the important, if not the sole, transmitters of human malaria and culicine mosquitoes as the vectors of avian malaria.

Recently, it has been demonstrated that such a relationship might not be as strict as once supposed, when *Anopheles quadrimaculatus* was shown to be susceptible to *Plasmodium lophurae* and *P. gallinaceum* (Coggeshall, 1941; Hurlbut and Hewitt, 1941, 1942; Haas and Akins, 1947). However, as the malaria infections upon which the anophelines were fed were in laboratory hosts, which differed considerably from the original hosts, the character of the plasmodium conceivably might have been altered by these experimental hosts. So the question as to whether this represented the true situation in nature remains unanswered.

Experiments having more bearing on the natural situation were done by Mayne (1928) and Lucena (1938). Mayne found some oöcysts in seven out of 144 *A. subpictus* fed upon sparrows (species?) which had bird malaria provisionally identified as *P. praecox.* One mosquito showed a few sporozoites in one gland. Lucena reported finding one oöcyst in *A. strodei* which had been fed upon a tico-tico (*Brachyspiza pileata*), harboring *P. cathemerium*(?). However, the paucity of observations in these experiments still left in considerable doubt the relative ability of anophelines to become infected with avian malarias in nature.

The recent report of Sabrosky, McDaniel and Reider (1946) showing that *A. crucians* in South Carolina had a relatively high sporozoite rate, led us to investigate the susceptibility of anophelines to local bird malarias. To determine the prevalence of such malarias, a survey of local birds extending over a period of several years was made (Hunninen and Young, 1950). This indicated that the most prevalent plasmodium was *P. relictum*, which was present quite commonly in English sparrows, *Passer domesticus. P. relictum* infections in these birds was chosen as the point of attack on the problem and the results obtained are reported here.

MATERIALS AND METHODS

The original sources of the *P. relictum* used in these experiments were three English sparrows, one of which was used in the feeding experiments (Sparrow 227). The infections were transferred from wild birds to canaries and to other sparrows by the injection of citrated blood intramuscularly. Transfer from canary to canary was both by infected blood and by sporozoites.

The mosquitoes used were *A. quadrimaculatus*, *A. crucians*, and *Culex pipiens*; the

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latter was chosen as the control because it has been shown by various investigators to be susceptible to *P. relictum*. All of the mosquitoes came from laboratory colonies.

The birds were kept in screen cages in a screened room. All of the blood smears were stained for 30 minutes with 1 to 10 dilution of Giemsa, to which one per cent Triton X-30 had been added.

For mosquito feedings, feathers were removed from the breast of the bird which was then placed in a holder made of $\frac{1}{4}$-inch wire cloth. The immobilized bird was

TABLE 1

Results of simultaneous feedings by different species of mosquitoes upon English sparrows infected with Plasmodium relictum

SPARROW NUMBER	*Culex pipiens*					*Anopheles quadrimaculatus*					*Anopheles crucians*				
	Days after feeding	Number dissected	Infected			Days after feeding	Number dissected	Infected			Days after feeding	Number dissected	Infected		
			Guts	Glands	Total			Guts	Glands	Total			Guts	Glands	Total
300	13	2	2	2	2	13	5	4	1	4	13	6	1	0	1
59	14	18	4	4*	4	14	7	3	0	3	15	6	0	0	0
59	15	12	3	1	3	15	13	5	0	5	15	5	1	0	1
300	16	7	6	6	6	16	6	6	0	6	15	2	1	0	1
243	17	11	2	3	3	19	3	0	0	0	19	1	0	0	0
296	26	35	0	9	9	18	4	0	0	0	16	7	0	0	0
258	15	9	1	1	1	15	5	1	1	1		0			
227	17	7	7	7†	7	17	7	4‡	1‖	4		0			
258	17	7	0	1	1	17	10	3§	1	3		0			
Total.............		108	25	34	36		60	26	4	26		27	3	0	3
Per cent Infected.......			23.2	31.5	33.3			43.3	6.7	43.3			11.1	0.0	11.1

* Sporozoites from glands injected intramuscularly into Canary 241 which did not become infected; examined over a period of 82 days.

† Sporozoites from glands of five mosquitoes injected intramuscularly into Canary 269 which did not become infected; examined over a period of 23 days.

‡ Sporozoites from gut injected into Canary 8 which did not become infected; examined 31 days.

§ Sporozoites from glands injected into Canary 270 which did not become infected; examined 60 days.

‖ Sporozoites from gut injected into Canary 288 which did not become infected; examined 57 days.

hung inside the cage containing the species of mosquitoes to be tested. In most of the cases the feeding was started at 4 p.m. and continued to 8 p.m. Twenty-four hours later the mosquitoes were taken from the feeding cage, placed in small plastic jars, and kept in the insectary (76°F. ± 2° and high relative humidity) until time for dissection. Dissections were made from 13 to 26 days after the infective feeding but usually on the 17th day. Twenty-four per cent of the *Culex*, 60 per cent of the *A. quadrimaculatus*, and 66 per cent of the *A. crucians* died before dissections were made. We were successful, although with difficulty, in getting about 200 *A. crucians*

to feed, but their large mortality rate after feeding explains the smaller number dissected.

RESULTS

Mosquitoes infected upon sparrows. Table 1 shows the results of the simultaneous feedings upon English sparrows infected with *P. relictum.* In experiments where the three mosquito species were fed together (the first six feedings), the *A. quadrimaculatus* showed the highest percentage (47.4 per cent) of guts infected with oöcysts, *C. pipiens* had 20.0 per cent infected, and *A. crucians* the least, 11.1 per cent. *C. pipiens* had the highest rate (29.4 per cent) of glands showing sporozoites, *A. quadrimaculatus* had 2.6 per cent glands positive, but none of the 27 *A. crucians* glands was positive.

TABLE 2

Results of simultaneous feedings by different species of mosquitoes upon canaries infected with Plasmodium relictum

CANARY NUMBER	*Culex pipiens*					*Anopheles quadrimaculatus*					*Anopheles crucians*				
	Days after feeding	Number dissected	Infected			Days after feeding	Number dissected	Infected			Days after feeding	Number dissected	Infected		
			Guts	Glands	Total			Guts	Glands	Total			Guts	Glands	Total
108	13	6	2	2	2	13	3	0	0	0	13	2	0	0	0
297	17	12	1	1	1	17	13	0	0	0	17	1	0	0	0
271	17	27	0	3	3	17	2	0	0	0	17	1	0	0	0
11046	18	10	1	1*	1	18	8	1	0	1	18	3	0	0	0
11046	19	6	2	2†	2	19	10	0	0	0	19	4	0	0	0
348	27	27	0	3	3	18	2	0	0	0	18	4	0	0	0
6	16	12	2	2	2	17	12	0	0	0		0			
11046	18	16	1	1‡	1	18	3	0	0	0		0			
Total.............		116	9	15	15		53	1	0	1		15	0	0	0
Per cent Infected.......			7.8	12.9	12.9			1.9	0.0	1.9			0.0	0.0	0.0

* Sporozoites from glands injected intramuscularly into Canary 6 which became infected on 8th day.

† Sporozoites from glands injected intramuscularly into Canary 38 which became infected on 9th day.

‡ Sporozoites from glands injected intramuscularly into Canary 22 which became infected on 8th day.

Of the three *A. crucians* infected, one had a single degenerating oöcyst; in another, 75 oöcysts were found, mostly degenerated; the third mosquito showed four oöcysts with faint striations indicating sporozoite development. No fully developed sporozoites were present in any of the oöcysts in *A. crucians.*

Comparing the totals where *A. quadrimaculatus* and *C. pipiens* were fed simultaneously, the former had a higher infection rate (an infection being defined as

oöcysts on the guts, and/or sporozoites in the glands) than the latter. *A. quadrimaculatus* had a higher proportion with infected guts and *C. pipiens* had a higher proportion showing sporozoites in the glands.

Of 26 *A. quadrimaculatus* that developed oöcysts, four also contained sporozoites in the glands. It is likely that others would have developed gland sporozoites had a longer time been allowed for development, as some of those dissected on the 14th, 15th, and 16th day (Sparrows 59 and 300) had oöcysts containing large numbers of motile sporozoites. Each of the companion lots of *C. pipiens* revealed some mosquitoes with gland sporozoites, indicating faster development of the plasmodium in this mosquito.

Mosquotoes infected upon canaries. Five canaries which had been infected by the intramuscular injection of infected blood, and one which had been infected by sporozoites originating from mosquitoes fed upon another canary, were used for simultaneous feedings of the three species of mosquitoes. The results are shown in table 2.

None of the 15 *A. crucians* was infected. Only one of the 53 *A. quadrimaculatus* (1.9 per cent) was infected and this was of low intensity; none showed sporozoites in the glands. On the other hand, *C. pipiens* showed 12.9 per cent with sporozoites in the glands, and some of these still had oöcysts on the gut.

Transmission experiments with sporozoites. Attempts to transmit the infections to canaries by injecting intramuscularly sporozoites from oöcysts and glands are summarized at the bottom of tables 1 and 2.

None of the five canaries receiving sporozoites from *C. pipiens* or *A. quadrimaculatus* that had fed upon sparrows developed an infection. However, all of the canaries that received sporozoites from *C. pipiens* that had fed upon canaries developed infections within eight to nine days.

DISCUSSION

The results of these experiments show that under certain conditions, *A. quadrimaculatus* can become infected with malaria present in native birds. Although in the early stages of development the anophelines showed a higher incidence of infection than the *C. pipiens*, in the mature developmental stage of sporozoites in the salivary glands, *C. pipiens* had a higher rate. However, it is considered important that 6.7 per cent of the *A. quadrimaculatus* had sporozoites in the glands.

A. crucians also was susceptible to bird malarias, although only three were infected. Because of the small number of *A. crucians* tested, a comparative evaluation is not attempted here.

The development of the parasite was slower, less uniform, and the infection was of lesser intensity in *A. quadrimaculatus* than in *C. pipiens*. There was a greater tendency for oöcysts to be abnormal and degenerate in the former. These facts, coupled with the greater number of infections reaching maturity in the latter species, indicates that *A. quadrimaculatus* was a less suitable host than *C. pipiens* under the conditions of the experiment.

Of particular interest was the apparent influence of the canary upon the *plasmodium* parasite. Each of the five attempts to pass the infection from sparrow to

canary by sporozoites from the mosquitoes failed. But once the infection had been established in the canary by blood inoculation, each of three attempts to transmit by mosquitoes to another canary was successful. This is similar to the results of Redmond (1944) who found that *P. relictum* of the pigeon could be established in canaries by blood transfer and transmitted by *C. pipiens* thereafter from canary to canary but not from canary to pigeon.

Furthermore, both *culex* and anopheline mosquitoes were less susceptible to the malaria in the canary than in the sparrow. Such results indicate a definite influence of the host upon the parasite and also show that in some cases canaries might be inadequate test animals to demonstrate the ability of mosquitoes to transmit malaria infections.

These results indicate quite clearly that *A. quadrimaculatus* can develop infections of avian malarias from native birds. Presuming that anopheline mosquitoes take some blood meals from English sparrows, it is obvious that such findings might have important bearings on the assumed incidence of human malaria based upon finding infected anophelines. There is a clear need for determining additional host-parasite relationships, such as the relative susceptibilities of various culicine and anopheline mosquitoes to the avian malarias commonly present in native birds, the preference of the mosquitoes for host blood meals, and the ability of the various malarias to develop in the mosquito under natural conditions.

SUMMARY AND CONCLUSIONS

1. *Plasmodium relictum* in wild-caught English sparrows was infective to *Anopheles quadrimaculatus*, *A. crucians*, and *Culex pipiens*. Complete development of the parasite occurred in both *A. quadrimaculatus* and *C. pipiens* but more frequently in the latter. *A. quadrimaculatus* showed a higher rate of infections on the gut than did *C. pipiens*.

2. The finding that anopheline mosquitoes can be readily infected by malarias in native birds is of considerable importance in interpreting data on the incidence of human malaria based upon mosquito dissections.

3. Mosquitoes did not transmit the infection from sparrow to canary. But when the infection was induced in the canary by blood inoculation, mosquitoes transmitted the infection from canary to canary. The infections in canaries were less infective to mosquitoes than were the infections in English sparrows.

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RESUMEN Y CONCLUSIONES

1. *Plasmodium relictum* tal como se encontró en gorriones ingleses que fueron capturados de la naturalez fué infectivo a *Anopheles quadrimaculatus*, *A. crucians* y *Culex pipiens*. Completo desarrollo del parásito ocurrió tanto en *A. quadrimaculatus* como en *C. pipiens*, pero más frecuentemente en este último. *A. quadrimaculatus* mostró un grado más alto de infección en el estómago que *C. pipiens*.

2. El hallazgo de que los mosquitos anophelinos pueden ser fácilmente infectados con malaria de las aves nativas es de considerable importancia en la interpretación de los datos de incidencia de malaria humana basados en disecciones de mosquitos.

3. Los mosquitos no transmitieron la infección de gorrión a canario, pero cuando la infección fué inducida en el canario por inoculación de sangre los mosquitos transmitieron la infección de canario a canario. Las infecciones de los canarios fueron menos infectivas a los mosquitos que las infecciones de los gorriones ingleses.

TRANSMISSION OF *HAEMOPROTEUS COLUMBAE* BY BLOOD INOCULATION AND TISSUE TRANSPLANTS

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Very little research has been done on the life cycle of the parasites of the genus *Haemoproteus*. The lack of an efficient and simple method of transferring the infection from bird to bird is one of the obstacles in the study of this group. Since only the gametocytes are found in the circulating blood it has been generally believed (Anschütz, 1909; Gonder, 1915, Aragão, 1916) that transmission of the infection by blood inoculation is not possible. Some workers have also tried to transmit *Haemoproteus* by the passage of organs or organ emulsions (Gonder, 1915; O'Roke, 1930; Coatney, 1933) with varying degrees of success. Most of these investigations have been carried out during the patent period of the infection in the donor. In our studies, primarily aimed at elucidation of the preerythrocytic stages of *Haemoproteus columbae*, we have obtained results with subinoculation of blood and tissues during the prepatent period which may lead to new points of view with regard to this parasite.

Young White Carneaux pigeons received from the Palmetto Pigeon Plant (Sumter, South Carolina) were used in our studies. Before any bird was used experimentally periodical blood examinations were made to show that it was free of blood parasites. Donor birds were infected with *Haemoproteus columbae* by the intramuscular injection of sporozoites, developed in pigeon-flies (*Pseudolynchia canariensis* Macquart) which had been allowed to live for at least two weeks in specially designed cages attached to gametocyte-carrying pigeons. The salivary glands of such pigeon-flies were prepared for injection by comminution in a 1:1 mixture of chick serum and physiological saline.

Subinoculations from 10 donor pigeons will be reported. Six were utilized for blood transfers on the 4th day of the prepatent period and one each on the 8th, 12th, 14th and 26th days (the day of sporozoite inoculation being designated day 0). Seven of the above birds were used as donors for tissue transplants; one additional bird, on the 20th day of the prepatent period, was used as a donor of lung tissue alone. In all, 38 recipients were required. Before a donor bird was sacrificed, about 10 ml. of blood were drawn from a wing vein into a syringe which had been washed with heparin solution. This blood was then introduced into a clean pigeon, one half being injected intravenously and the other half intraperitoneally. The donor pigeon was then killed by pressure on the neck, and pieces of its lung, spleen, liver, heart and brain were transplanted separately into five healthy birds. The recipients were anesthetized with nembutal. The transplants of each tissue, consisting of from three to eight pieces (each about 5 cu. mm.) were placed either in the pectoral muscles or in the peritoneal cavity. Blood smears from the recipients were then examined daily for 60 days, beginning 8 to 14 days after day 0.

Successful subinoculations of blood were obtained in four of the six instances where

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blood was drawn on the 4th day of the prepatent infection. On the other hand, the recipients of blood drawn from 8-, 12-, 14- and 26-day old infections remained negative. Details of the infected recipients follow:

Pigeon 15 exhibited gametocytes in the blood 36 days after inoculation. The infection was low-grade but persisted until the bird was sacrificed 60 days after inoculation.

Pigeon 64 developed a moderately high parasite density in the blood which began 31 days after the blood inoculation and continued until the bird was killed on day 60.

Pigeon 21 first showed gametocytes 40 days after the blood inoculation, and a low-grade infection persisted until the bird was sacrificed on day 60.

Pigeon 73 showed parasites in the blood 33 days after the inoculation. This bird is still under observation and showing a moderately high gametocyte count 50 days after inoculation.

Of the two birds which failed to develop parasitemia one, pigeon 63, received blood from the same donor as did pigeon 64 above, but only one-third the volume was given. Both the negative birds were kept under observation for 60 days following the blood inoculation.

Nine birds were used as recipients of lung tissue transplants (three taken on day 4, two on day 8 and one each on days 12, 14, 20 and 26 of the prepatent period). Three became infected: pigeon 78, given lung tissue obtained on the 14th day of a prepatent infection, showed gametocytes 25 days after operation; pigeon 93, given lung tissue from a 20-day old infection, showed gametocytes 21 days after operation; a pigeon 89, given lung tissue from a 26-day old infection, showed gametocytes 21 days after operation. In all cases the parasite levels in the recipients were low-grade but persistent. The birds given lung tissue from prepatent infections which were 4, 8 and 12 days old did not become infected.

None of the recipients of spleen, liver, heart and brain transplants exhibited parasitemia during 60 days of observation.

SUMMARY

Haemoproteus columbae infections in pigeons were transmitted in four of six trials by the transfer of 10 ml. of blood from donor birds which had been inoculated with sporozoites four days previously. Single trials at 8, 12, 14 and 26 days were unsuccessful. Transplants of lung tissue were infective when taken from 14-, 20- or 26-day old prepatent infections, but were negative at 8, 12 and 14 days. Transplants of spleen, liver, heart and brain from the above pigeons were not infective.

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PLANT CONTROL STUDIES IN TENNESSEE VALLEY RESERVOIRS

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Phytocidal studies on the use of synthetic plant hormones such as 2,4-D (2,4-dichlorophenoxyacetic acid and derivatives) for the control of littoral plants for malaria control were initiated during the 1945 season. These initial studies were progressively expanded and extended through the 1949 season. The scope of the studies is reflected by the treatment of more than 500 field plots in the Kentucky, Pickwick, Wilson, Wheeler, Guntersville, and Norris Reservoir areas, and the analyses of more than 8,000 sampling quadrats involving field counts in excess of 150,000 plants. It is the purpose of this paper to summarize observations made during the study period through the 1949 season.

EXPERIMENTAL STUDIES

1. *General Observations*

Studies showed that 2,4-D was taken up by stems, roots, and leaves and each of these organs proved to be good portals of entry for the phytocide. In general, the active principle was found to move more readily upstem than downstem using the semaphore-like response of leaves as an indicator. Translocation of the active principle was demonstrated more readily in plants with well-developed vascular systems, such as black willow, than in submersed species with reduced vascular systems, such as water milfoil. Information from these types of observations supplemented by spray tests helps to define objectives in the field operation. For example, they indicate that: (1) complete coverage of susceptible plants with the spray is desirable for the most effective results, (2) plants such as alligator weed should be dewatered or nearly so before treatment to keep the necessary downward distance of transport to a minimum and thereby increase the possibility of the active principle reaching the roots, and (3) treatment of submersed species will probably be followed by little or no control even though the spray is applied when the plants intersect the water surface.

During the course of the studies, a number of interesting formative effects were produced by plants subjected to sublethal dosages of 2,4-D and related compounds (Figure 1.) These formative effects involved roots, stems, leaves, flowers, and fruits. Adventitious roots or root-like structures were produced along the internodes of stems of alligator weed and Spanish needles. Stems of several composites commonly assumed an S-curvature following exposure to the hormones. Unusual effects on leaves included the development of pig-tail coils in the petioles of lotus as well as abnormal blades. A large number of species produced unusual leaf blades following exposure and these included stinkweed, cocklebur, cotton, grape, trumpet vine, and others. In some species, such as zinnia and buttonball, opposite leaves fused to form

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FIG. 1. Some formative effects of 2,4-D on plants

(a) Lateral fusion of opposite leaves of button weed (*Diodia virginiana*).
(b) Fusion of opposite leaves of garden zinnia (*Zinnia* sp.) forming a cup.
(c) Dichotomous forked leaf of black willow (*Salix nigra*) on alternate leaf species.
(d) "Buggy seat" type of leaf produced by lotus (*Nelumbo pentapetala*).
(e) Tubular ray flowers produced by sneezeweed (*Helenium nudiflorum*).
(f) "Jap hat" type of leaf produced by lotus (*Nelumbo pentapetala*).

a cup-like structure. In general, perennial species in which opposite leaves fused were relatively difficult to control with the hormone sprays. Forked blades were observed to develop in the opposite-leaved buttonweed and also in the alternate-leaved black

willow. Formative effects developed in black willow, buttonball, trumpet vine, green ash, wild cotton, and grape at least one year after exposure of the vegetative bodies to sublethal dosages of 2,4-D. Flower modification was observed in sneezeweed which produced tubular ray flowers in contrast to the flat ones observed on normal plants. In some annual species, such as ragweed, and in some perennial species, such as buttonball, fruiting was prevented. The fact that hormones can prevent the production of seed in dosages sublethal to the vegetative body suggests that a plant, such as water chestnut, in which the seeds are known to remain viable only one year, might be effectively controlled by the deliberate application of sublethal dosages to inhibit fruiting. In cocklebur, formative effects were carried over the winter in the seed and abnormalities appeared in the seedling on germination the following year.

2. *Volume of Spray*

During the 1945 season it was shown that single drops of hormone concentrates would induce physiological activity in plants. These results suggested the use of concentrated sprays of low volume and accordingly preliminary spray treatments were made to lotus, wild cotton, alligator weed, and other plants with encouraging results. During the 1946-1949 seasons the studies were expanded and the possible methods of application explored in detail on more than 30 species of plants. Applications of sprays and/or dusts have been made by hand equipment, by ground and boat mechanized equipment, and by airplane. These studies have demonstrated that littoral plants can be killed by the use of low volumes of highly concentrated hormone sprays.

One of the most important factors in securing effective control with hormone sprays appears to be that almost complete coverage of the plant in full summer foliage by the spray is required. This coverage may be obtained either by coarse high volume sprays of low concentration or by fine low volume sprays of high concentration. As a general rule, at least on herbs, similar control results should be anticipated with a recovery rate of one pound 2,4-D per acre whether it be applied in dilute solution at a rate of about 125 gallons per acre or as a high concentrate at a rate of about one quart per acre provided that in each instance the particle size is adjusted so as to "wrap the plants up in the spray." For the control of woody species by ground application, high volume sprays of about 100 gallons per acre have given better results than low volume sprays. However, low volume sprays applied by airplane at a rate of about one quart per acre have given effective results on woody species, presumably due to the better breakup and better coverage associated with this method of application. Observations indicated that better results were obtained on woody species with one quart of spray per acre applied by plane than 25 gallons of spray per acre discharged by a ground spray boom at equal 2,4-D acid rates per acre. These observations all appear to emphasize the importance of complete coverage of the plants for effective control. In general, as the volume of the spray per acre is decreased, the particle size of the spray must also be decreased to insure good coverage of the plant. This decrease in volume and particle size results in an increase in drift hazard. Therefore, in working near sensitive crop plants, a higher volume coarse spray would be favored over a lower volume mist spray. Where sensitive crop plants

are not involved, low volume mist sprays would be favored except for ground application to woody species.

3. *Season of Treatment and Growth Stage*

Area treatment of plots with a calibrated spray-boom unit during winter, spring, summer, and fall has led to the general conclusion that many species of plants are most susceptible to 2,4-D sprays when applications are made during the growing season after full summer foliage is reached. Cattail was a notable exception to this general rule since it was found to be more susceptible during its spring growth period than at other seasons or during other growth stages.

Observations have suggested that the growth stage or phenological aspect of the plant at the time of treatment, independent of season, is one of the most important factors with respect to the control effectiveness of hormone sprays. For example, the foliage treatment of actively sprouting stumps of willow in midsummer when they are in the spring phenological aspect has yielded poorer control than fall treatment of the more advanced growth in the summer aspect. Similarly, treatment of stumps while dormant in midwinter or in midsummer with area sprays has yielded little or no control. Other workers have developed special techniques and have secured good control with stump spraying during the dormant season using about four per cent sprays applied on the stump surface.

The development of early spring and fall treatments is desirable as these periods coincide with the off-season for cotton and other sensitive crop plants. A number of species may be killed by foliage sprays in the fall, but cattail is the only species found to be most susceptible during its early spring growth period.

4. *Comparison of Formulations*

Studies were conducted in an attempt to compare the relative effectiveness of various 2,4-D formulations. Black willow, buttonball, trumpet vine, lotus, cocklebur, lizard tail, and cattail were used as the test plants (Tables 1 and 2). Formulations used included the methyl, ethyl, isopropyl, butyl, and amyl esters of 2,4-D and the sodium, triethanolamine, and morpholine salts of 2,4-D. Some of the earlier plots were treated by pump-up can and wide variations in the results from some of the replicated plots led to the conclusion that this variation reflected the unevenness of hand distribution. Later a calibrated spray boom unit was used on the assumption that uniform treatment would insure uniform results. However, even with the calibrated spray unit, considerable variation continued to be experienced in a large percentage of the replicated plots and thus uniform treatment did not insure relatively uniform results. It appears that much of the variation may be attributed to differences in growth stages of the plants at the time of treatment due either to differences in cutting dates previous to treatment or to differences in contour location of treatment plots. Unfortunately, these plots were set out previous to the recognition of the importance of the growth stage at the time of treatment. Therefore, it is not possible to establish the formulation as the limiting factor in these series of treatments since the wide variation in some of the replicated plots shows that often some other factor or combination of factors becomes limiting.

TABLE 1

*Effectiveness of Various Formulations of 2,4-D Applied by Pump-Up Can to Various Marginal Species at Rates of 1 and 3 lbs. 2,4-D Per Acre**

Treatments Applied to Plots in Duplicate at Each Rate. Data based on analysis of 1920 quadrats. Plots treated during June and July, 1946.

2,4-D FORMULATION AND TIME OF EVALUATION	PER CENT CONTROL†															
	Lotus				Lizard Tail				Cocklebur				Black Willow			
	lbs./A				lbs./A				lbs./A				lbs./A			
	1		3		1		3		1		3		1		3	
	Av.	Range	Av.	Range	Av.	Range	Av.	Range	Av.	Range	Av.	Range	Av.	Range	Av.	Range
½% Sodium salt																
Same season	79	76–81	84	72–96	0	0–0	4	0–7	97	96–97	100	100–100	3	0–5	18	16–19
One year	1	0–1	7	0–14	23	0–45	66	65–66	77	67–87	57	44–69	25	17–33	59	45–72
½% Trisalt																
Same season	79	70–88	89	88–91	12	6–18	3	0–5	88	82–94	99	97–100	20	15–25	56	41–70
One year	34	18–49	38	14–61	39	36–41	4	0–8	27	7–46	27	0–54	47	32–62	18	2–33
½% Methyl ester																
Same season	97	94–99	99	99–100	17	0–33	22	0–41	83	69–95	99	97–100	22	3–40	58	30–85
One year	98	95–100	96	91–100	41	26–55	19	3–35	26	0–52	18	13–22	38	32–44	55	42–68
5% Methyl ester																
Same season	93	87–98	100	100–100	0	0–0	15	0–30	64	49–78	99	99–100	25	13–36	32	13–48
One year	50	0–100	56	15–97	20	4–35	18	0–35	14	8–19	41	38–43	23	5–40	25	9–40
4% Butyl ester																
Same season	92	88–95	100	100–100	0	0–0	0	0–0	72	55–88	97	95–99	18	1–35	36	26–46
One year	90	88–91	97	94–100	24	0–47	0	0–0	54	43–64	68	58–77	21	2–40	31	21–40
Untreated check																
Same season	0		0		0		0		20		20		0		0	
One year	12		12		0		0		45		45		0		0	

* Or butyl ester 1 and 3 lbs. per acre.

† Per cent control is given to nearest whole per cent except that all averages between 99 and 100 per cent are listed as 99 in order to show instances of incomplete control.

From the results obtained it appears that there might be some small differences in the phytocidal properties of the different 2,4-D formulations on the species used as test plants. However, it is felt that such differences, if real, are mainly of academic

TABLE 2

Effectiveness of Various Formulations of 2,4-D Applied by Calibrated Command Car Unit to Various Marginal Species at a Rate of 1 lb. (½%) 2,4-D Per Acre and 25 Gallons of Spray Per Acre

Data based on analysis of 1810 quadrats. Plots treated during July and August, 1947.

2,4-D FORMULATION AND TIME OF EVALUATION	PER CENT CONTROL							
	Black Willow		Trumpet Vine		Buttonball		Lizard Tail	Cattail
	3 Plots		2 Plots		3 Plots		Single Plots	Single Plots
	Av.	Range	Av.	Range	Av.	Range		
Amyl ester								
Same season	40	29–51[a]	37[b]		15	2–27[a]	35	35
One year	25	22–28	54[b]		0	0–0[c]	0	97
Butyl ester in kerosene								
Same season	56	27–87	34	22–46	31	20–39	1	32
One year	55	53–60	35	21–50	1	0–5	8	94
Butyl ester emulsion								
Same season	59	42–91	56	40–71	24	0–39	12	57
One year	49	25–67	38	35–44	0	0–0	0	96
Ethyl ester								
Same season	40	31–48	42	27–56	9	1–18	0	50
One year	36	15–54	55	51–59	2	0–4	20	90
Isopropyl ester								
Same season	23	11–28	36	30–42	8	1–18	0	36
One year	21	0–33	58	54–61	0	0–0	20	98
Morpholine salt and wetting agent								
Same season	55	34–90	33	28–38	10	7–15	0	28
One year	27	6–57	42	41–43	0	0–0	0	97
Morpholine salt								
Same season	56	37–92	58	33–82	20	19–20[a]	3	54
One year	36	16–56	45	35–55	1	0–2	25	99
Sodium salt								
Same season	43	34–60	45	31–59	14	4–32	0	56
One year	33	21–44	65	62–69	1	0–1	12	96
Check plots								
Same season	3	0–10[d]	1	0–4[c]	0[b]		0[a]	12
One year	8	0–43[e]	18	0–38	0[d]		4(13–0)[d]	95[d](87–100)

a = 2 plots. b = 1 plot. c = 3 plots. d = 4 plots. e = 5 plots.

interest as variations in results with the same formulation were often greater than differences between formulations, and actual control obtained at one year, for some species at least, contradicted the appraisal made the same season of application. Nevertheless, there was some indication in this series of applications that the following formulations were slightly more toxic to the species indicated.

Black willow—methyl ester, butyl ester, ethyl ester, and morpholine salt
Trumpet vine—sodium salt, isopropyl ester, ethyl ester, and amyl ester
Lizard tail—sodium salt
Cattail—butyl ester, sodium salt, morpholine salt, and ethyl ester
Lotus—all formulations of salts and esters tested gave good leaf kills but plots treated with esters showed little or no resprouting, whereas those treated with salts commonly resprouted but not in all cases. Dust and pellet formulations of 2,4-D have given excellent results on lotus at application rates of about two pounds acid equivalent per acre.

A comparison of data for all plots treated with salts and with esters at one pound, 2,4-D acid equivalent rate per acre was made. The combined data indicated that there was no significant difference between salts and esters for the control of black willow, buttonball, trumpet vine, cocklebur, lizard tail, and cattail. However, on lotus the esters appeared to be favored for control although both esters and salts gave similar leaf kills the same season of application. The similar toxicity of the 2,4-D salts as a group versus the 2,4-D esters to the test plants involved indicates that in general the 2,4-D acid equivalent rate per acre is the controlling factor rather than the formulation. From these studies, the use of a 2,4-D salt is recommended as salts are more economical and have the added advantage of being relatively fumeless as compared to the more costly esters.

TABLE 3

Spring Treatment of Cattails by Knapsack Sprayer in Similar Growth Stages

FORMULATION	PER CENT CONTROL SAME SEASON OF APPLICATION	
	Cattail (3 plots)	Black Willow (Single Plots)
Butyl Ester Emulsion	55	90
Isopropyl Ester	15	53
Morpholine Salt	15	65
2,4,5-T	21	30
Check	16	0

Supplementary hand-sprayed plots in triplicate for cattail and single plots for willow were treated with four hormone formulations during the spring of 1948. In these series, applications were made at a rate of three pounds acid equivalent per acre and the plots were appraised the same season of application. In both treatment and appraisal, attention was given to colonies at similar contours and hence plants were in similar growth stages at the time of and after treatment. The results are given in Table 3.

The results of the spring treatments indicate some differences in the initial action of the different formulations on cattail and black willow and suggest that the butyl ester formulation might have more phytocidal action than the other formulations on these two species. Fall treatment of cattail at three pounds 2,4-D acid equivalent per acre with the butyl ester gave little or no control.

The airplane treatment of a colony of cattail with the butyl ester concentrate in the spring of 1949 gave excellent top kills. Another colony of cattail was treated in the spring with a mixture of the butyl ester of 2,4-D and 2,4,5-T concentrate and in this instance little or no control resulted. Both treatments were made at similar rates and at about the same time. The plants of which the tops were killed were in an earlier stage of development than those which showed little or no control in response to the treatment.

Studies with the use of sodium salt of 2,4-D have shown that the species listed below can be controlled, as evaluated one year after treatment, at an application rate of three pounds of 2,4-D acid equivalent or less per acre (Table 4).

Water primrose
Water willow weed
Wild cotton
Weak rush
Giant ragweed
Cocklebur
Black willow
Trumpet vine

Where comparisons have been possible with salts and esters for the foregoing species, the results with the 2,4-D salts have compared favorably. The effectiveness of 2,4-D phytocides for controlling six species of plants is shown in Figure 2.

5. *Special Studies on Buttonball*

Observations demonstrated that the rate of three pounds acid equivalent of 2,4-D per acre was not adequate to control buttonball. Therefore various formulations listed in Table 5 were applied at the rates of six and twelve pounds of acid equivalent per acre in an effort to control this species. In some cases mixtures of 2,4-D and 2,4,5-T were used, and in these instances equal acid equivalents were employed in the spray. The results from these tests showed that none of the formulations gave any practical control (Table 5). In general, the degree of control obtained at six pounds acid equivalent was as effective as that at the twelve pound rate. Better results were obtained with mixtures of the butyl ester 2,4-D and isopropyl ester 2,4,5-T, the isopropyl ester 2,4-D and 2,4,5-T, and the straight isopropyl ester of 2,4-D. In this series of tests the butyl ester, morpholine salt, pentachlorophenol, and mixtures of the morpholine salt and pentachlorophenol gave essentially no control of buttonball even though all gave good leaf kills the same season of application.

6. *Minimum Lethal Dosage*

The minimum lethal dosage of 2,4-D for lotus was determined using five esters and four salts (Table 6). Evaluations of leaf kills obtained the same season of application indicated that the minimum lethal dosage of all formulations was in the neighborhood of $1\frac{1}{2}$ ounces 2,4-D acid equivalent per acre as predicted from other field tests. Similar results were obtained with 2,4,5-T on lotus. Careful checks were not possible one year after treatment due to the treatment area being dewatered. Detailed

TABLE 4

Effectiveness of Hand Applications of a ½ Per Cent Solution of the Sodium Salt of 2,4-D Applied at Rates of 1 and 3 lbs. 2,4-D Per Acre to Various Marginal Plants

Data based on analysis of 1730 quadrats.* Plots treated during June and July, 1945.

SPECIES	TIME OF EVALUATION AFTER TREATMENT	PER CENT CHANGE IN ORIGINAL POPULATIONS AFTER TREATMENT		
		1 lb. 2,4-D/A	3 lbs. 2,4-D/A	Untreated
Buttonball	Same season	−6	−33	−10
	One year	−45	−53	−34
Trumpet vine	Same season	−94	−100	−17
	One year	−61	−62	−41
Cowlily	Same season	−46	−33	−25
	One year	−25	−16	+12
Water primrose	Same season	−7	+15	−46
	One year	−96	−97	+6
Willow weed	Same season	+31	−25	+27
	One year	+44	−42	+25
Burhead	Same season	−99	−99	0
	One year	−18	+18	−25
Wild cotton	Same season	−59	−87	+100
	One year	−99	−99	+107
Cattail	Same season	+51	+58	+26
	One year	+28	+100	+50
Giant cutgrass	Same season	+8	+25	+4
	One year	−4	+40	−5
Weak rush	Same season	−53	−65	+1
	One year	−60	−62	+11
Weak rush†	Same season	−50	−60	+1
	One year	−30	−100	+11
Woolgrass	Same season	+15	+22	+29
	One year	+76	+32	+108
Giant ragweed	Same season	−98	−100	−35
	One year	−7	+11	0
Panic grass	Same season	+24	+15	+23
	One year	+15	+12	0
Smartweed	Same season	+73	+79	+15
	One year	+40	+14	+35
Black willow‡ (seedlings 4–6′)	Same season	−97	−100	0
	One year	−99	−100	+3
Green ash§	Same season	+25	+20	+67
	One year	+50	+40	+100

* Per cent change is given to nearest whole per cent except that all averages between 99 and 100 per cent are listed as 99 in order to show instances of incomplete control.

† Received spring and summer treatment.

‡ $\frac{1}{10}$% solution of sodium salt 2,4-D.

§ Received spring treatment.

studies on the minimum lethal dosage of 2,4-D for other plants have not been made, but it appears that for most other susceptible species it is considerably higher and ranges from one to three pounds of 2,4-D per acre. For a so-called resistant species,

FIG. 2. Certain species of plants may be controlled readily with 2.4-D

(a) Cocklebur (*Xanthium americanum*).
(b) Black willow (*Salix nigra*).
(c) Soft rush (*Jancus effusus*).
(d) Lotus (*Nelumbo pentapetala*).
(e) Parrots feather (*Myriophyllum brasiliense*).
(f) Trumpet vine (*Campsis radicans*).

such as buttonball, observations show that the minimum lethal dosage for this species exceeds 12 pounds of 2,4-D acid equivalent per acre.

TABLE 5

Treatments on Buttonball Applied at Rates of 6 and 12 Pounds Acid Equivalent Per Acre at Spray Concentrations of $\frac{3}{4}$% and $1\frac{1}{2}$%

Treatments Made by Calibrated Command Car Unit at 100 Gallons of Spray Per Acre in Each Case. Evaluations One Year After Treatment. Data Based on Analyses of 43 Sixteen Square Meter (or larger) Quadrats. Plots treated during September and October, 1948.

FORMULATION	6 LBS. ACID EQUIV./AC. $\frac{3}{4}$% SPRAY-100 GALS./AC.		12 LBS. ACID EQUIV./AC. $1\frac{1}{2}$% SPRAY-100 GALS./AC.	
	% Dead	No. of Plots	% Dead	No. of Plots
Butyl Ester 2,4-D & Isopropyl Ester 2,4,5-T	16(12–21)	4	9(1–26)	6
Isopropyl Ester 2,4-D & Isopropyl Ester 2,4,5-T	11(4–19)	2	8(2–13)	2
Butyl Ester 2,4-D	0(0–0)	2	4(3–4)	2
Isopropyl Ester 2,4-D	2(0–5)	3	—	—
Isopropyl Ester 2,4,5-T	17(9–24)	2	—	—
Triethanolamine Salt 2,4-D	—	—	0(0–0)	4
Morpholine Salt 2,4-D	1(0–2)	2	4(0–8)	3
Morpholine Salt 2,4-D 1 lb. & Pentachlorophenol* 5 lbs.	0(0–0)	2	—	—
Morpholine Salt 2,4-D 6 lbs. & Pentachlorophenol* 6 lbs.	—	—	0	—
Pentachlorophenol* 12 lbs.	—	—	2(0–3)	2
Check Plots	1(0–1)	3	1(0–1)	3

* Pounds of Pentachlorophenol per acre.

TABLE 6

Effectiveness of Various 2,4-D Formulations on Lotus Applied at Rates of 1 lb. (2%), $\frac{1}{4}$ lb. ($\frac{1}{2}$%), and $\frac{1}{16}$ lb. ($\frac{1}{8}$%) Acid Per Acre and Dispersed by Pump-Up Can at Rate of 3 Gallons of Spray Per Acre

All plots in triplicate and evaluated same season of application. Data based on analysis of 1080 quadrats. Plots treated during July 1948

FORMULATION	PER CENT KILL*					
	1 lb./A		$\frac{1}{4}$ lb./A		$\frac{1}{16}$ lb./A	
	Average	Range	Average	Range	Average	Range
Methyl ester 2,4-D	96	95–98	93	84–98	69	42–88
Ethyl ester 2,4-D	98	97–99	92	81–98	71	36–89
Isopropyl ester 2,4-D	95	92–100	92	87–95	58	27–77
Butyl ester 2,4-D	95	92–98	90	82–95	69	33–94
Amyl ester 2,4-D	96	96–97	92	90–97	67	40–83
2,4,5-TCP	85	64–95	83	62–94	57	32–78
2,4,5 TCP + 2,4-D-IPE	97	96–99	87	80–91	63	37–80
Morpholine salt 2,4-D	95	94–95	92	91–94	60	24–80
Triethanolamine salt 2,4-D	91	80–97	89	82–97	58	22–85
Alkanolamine salt 2,4-D	87	81–92	92	87–95	65	50–74
Sodium salt 2,4-D	81	61–90	88	87–89	61	23–86
Check	18	15–20	17	13–23	24	11–36

* Per cent kill is given to nearest whole per cent except all values between 99 and 100 per cent are listed as 99 to show all instances of incomplete control.

7. *Synergism*

A mixture of the isopropyl ester of 2,4-D and 2,4,5-T in equal parts was applied to lotus (Table 6) and, likewise, each formulation was applied separately. Similar leaf kills resulted in each case and no evidence of synergism was observed using lotus as the test plant. Also, careful field tests on buttonball failed to show any significant evidence of synergistic action of the mixture.

Observations of mixed woody species sprayed with a mixture of esters of 2,4-D and 2,4,5-T by the North Carolina Tree Service near Goldsboro, North Carolina, showed remarkable kills to many woody species generally recognized to be resistant to 2,4-D. Subsequent large-scale field treatments by the Division of Power Operations of the Tennessee Valley Authority with a mixture of 2,4-D and 2,4,5-T also showed remarkable kills to many so-called resistant woody species. It appears that mixtures of 2,4-D and 2,4,5-T exhibit real synergistic action when applied to many woody plants. It is thought that the concentration of the mixture is also of importance in controlling so-called resistant woody species.

8. *Water vs. Kerosene as a Carrier*

Kerosene was used as a carrier for 2,4-D because it offered the apparent advantages of serving as a sticker and penetrant, particularly for waxy leaf plants such as lotus. However, results have indicated that there is no real advantage in the use of kerosene as a carrier even for such waxy leaved plants. For a plant such as alligator weed, kerosene has been used with a 2,4-D ester in an effort to increase control effectiveness with better results on coarse soils than on clay soils. In general, in the interest of economy and facility of application, water is preferable as a carrier and sprays may be dispersed either as emulsions or as aqueous solutions with excellent control results.

9. *Wetting Agent*

The addition of a wetting agent, such as Triton X-100, at one pound per 25 gallons of spray, appeared to have no special advantage (Table 2). In some instances, better results were obtained with the wetting agent added, and in other instances the spray minus the wetting agent gave better results.

10. *Susceptibility of Plants to 2,4-D*

A tentative classification has been made of the relative susceptibility of plants to hormone sprays applied at dosages up to three pounds 2,4-D per acre, using the degree of control obtained as the criterion of effectiveness. Following the suggestion of the North Central Weed Control Conference, four groups are recognized:

Hypersensitive: Species which may be killed in a single application in the susceptible growth stage.

Sensitive: Species which may be killed usually by two applications, under favorable conditions.

Semi-Tolerant: Species which may be set back by repeated applications under favorable conditions but continue to survive.

Tolerant: Species which show little or no effect from repeat applications.

TABLE 7

A Tentative Table of the Relative Susceptibility of Woody Plants to 2,4-D for Control as Based on Observations Carried out in the Tennessee Valley During the Summers of 1945 to 1948*

	Hypersensitive
River birch	*Betula nigra* L.
Trumpet vine	*Campsis radicans* Seerman.
Hazelnut	*Corylus americana* Marsh.
Tupelo gum	*Nyssa aquatica* L.
Sycamore	*Platanus occidentalis* L.
Black willow	*Salix nigra* Marsh.
	Sensitive
Pepper vine	*Ampelopsis arborea* (L.) Rusby.
Florida vine	*Brunnichia cirrhosa* Banks.
Sweet gum	*Liquidambar styraciflua* L.
Honeysuckle	*Lonicera japonica* Thunb.
Poison ivy	*Rhus toxicodendron* L.
	Semi-Tolerant
Box elder	*Acer negundo* L.
Silver maple	*Acer saccharinum* L.
Buttonball	*Cephalanthus occidentalis* L.
Persimmon	*Diospyros virginiana* L.
Green ash	*Fraxinus pennsylvanica* Marsh. var. *lanceolata* (Borkh.) Sarg.
Cottonwood	*Populus deltoides* Marsh.
Overcup oak	*Quercus lyrata* Walt.
Black locust	*Robinia pseudo-acacia* L.
Blackberry	*Rubus* sp.
Sassafras	*Sassafras albidum* (Nutt.) Nees.
	Tolerant
False indigo	*Amorpha fruticosa* L.
Red cedar	*Juniperus virginiana* L.
Loblolly pine	*Pinus taeda* L.
Scrub pine	*Pinus virginiana* Mill.
Bald cypress	*Taxodium distichum* (L.) Rich.

* Nomenclature after Muenscher, W. C., 1946. Keys to Woody Plants. Ed. 5, Comstock Publishing Co., Ithaca, New York.

The tentative classifications of 26 woody and 59 herbaceous species according to the above groupings are given in Tables 7 and 8.

11. *Effect of 2,4-D on Crop Plants*

Cotton is very sensitive to 2,4-D and formative effects appear in the plants after exposure to minute quantities of 2,4-D. Light drift on cotton in the seedling stage

TABLE 8

A Tentative Table of the Relative Susceptibility of Herbaceous Plants to 2,4-D for Control as Based on Observations Carried Out in the Tennessee Valley During the Summers of 1945 to 1948*

	Hypersensitive
Lesser ragweed	*Ambrosia elatior* L.
Giant ragweed	*Ambrosia trifida* L.
Ammannia	*Ammannia coccinea* Rottb.
Spanish needles	*Bidens aristosa* (Michx.) Britt.
Umbrella sedge	*Cyperus pseudovegetus* Steud.
Jimson weed	*Datura stramonium* L.
Swamp loosestrife	*Decodon verticillatus* (L.) Ell.
Square-stem spikerush	*Eleocharis quadrangulata* (Michx.) R. & S.
Horseweed	*Erigeron canadensis* L.
Cotton	*Gossypium* sp.
Wild cotton	*Hibiscus militaris* Cav.
Soft rush	*Juncus effusus* L.
Water primrose	*Jussiaea diffusa* Forsk.
Hairy water primrose	*Jussiaea grandiflora* Michx.
Water willow weed	*Justicia americana* (L.) Vahl.
Parrots feather	*Myriophyllum brasiliense* Camb.
American lotus	*Nelumbo pentapetala* Walt.
Toothcup	*Rotala ramosior* (L.) Koehne.
Cocklebur	*Xanthium americanum* Walt.
	Sensitive
Aster	*Aster* sp.
Wild cotton	*Hibiscus moscheutos* L.
Spiny waterleaf	*Hydrolea quadrivalvis* Walt.
Wild lettuce	*Lactuca scariola* L.
Water willow	*Ludvigia alternifolia* L.
Cowlily	*Nuphar advena* Ait.
Buckhorn plantain	*Plantago lanceolata* L.
Beaked rush	*Rhynchospora corniculata* (Lam.) Gray
Goldenrod	*Solidago altissima* Nutt.
Dandelion	*Taraxacum officinale* Weber.
	Semi-Tolerant
Burhead	*Echinodorus radicans* (Nutt.) Engelm.
Sneezeweed	*Helenium nudiflorum* Nutt.
Heliotrope	*Heliotropium indicum* L.
Water purslane	*Ludvigia palustris* (L.) Ell.
White waterlily	*Nymphaea odorata* Ait.
Green arum	*Peltandra virginica* (L.) Kunth.
Stinkweed	*Pluchea* sp.
Dock	*Rumex verticillatus* L.
Duck potato	*Sagittaria latifolia* Willd.
Lizard tail	*Saururus cernuus* L.
Bluestem	*Scirpus validus* Vahl.
Cattail	*Typha latifolia* L.

TABLE 8—*Concluded*

	Tolerant
Broomsedge	*Andropogon virginicus* L.
False-nettle	*Boehmeria cylindrica* (L.) Sw.
Hornwort	*Ceratophyllum demersum* L.
Buttonweed	*Diodia virginiana* L.
Wild millet	*Echinochloa crus-galli* (L.) Beauv.
Eclipta	*Eclipta alba* (L.) Hassk.
Rice cutgrass	*Leersia oryzoides* (L.) Swartz.
Water milfoil	*Myriophyllum heterophyllum* Michx.
Giant smartweed	*Polygonum coccineum* Muhl.
Smartweed	*Polygonum hydropiperoides* Michx.
Purslane	*Portulaca oleracea* L.
Woolgrass	*Scirpus cyperinus* (L.) Kunth.
Horse-nettle	*Solanum carolinense* L.
Johnson grass	*Sorghum halepense* (L.) Pers.
Bur-reed	*Sparganium americanum* Nutt.
Geramander	*Teucrium canadense* L.
Bladderwort	*Utricularia gibba* L.
Giant cutgrass	*Zizaniopsis miliacea* (Michx.) Doell. & Aschers.

* Nomenclature after Isely, D., 1946. Manual of Herbaceous Plants of the Tennessee Valley Reservoirs.

had no apparent adverse effect on the crop, but drift on plants in flower interfered with fruiting and damaged the crop severely. "Drift" from the airplane application of an ester produced formative effects in seedlings up to $1\frac{1}{2}$ miles from the treatment area in one instance, but in other instances esters have been applied by plane without any evidence of drift. Cotton seedlings exposed to light drift of 2,4-D remained green and healthy later in the dry part of the season, whereas unexposed fields of cotton appeared sickly and somewhat wilted. Because of the high sensitivity of cotton to 2,4-D, special care should be used in airplane application, and treatments should be made either during the off-season for cotton or only in areas safely removed from cotton fields.

12. *Other Herbicides*

Treatments were made with other herbicides such as isopropylphenyl carbamate (IPC), ammonium trichloroacetate (ATA), and ammonium sulfamate to several species (Table 9). The application of one pound per acre of IPC gave no evidence of control of established perennial grasses or other monocots. ATA applied at a rate of 100 pounds active material per acre gave essentially 100 per cent control of panic grass (*Panicum agrostoides*) for at least two years, but was ineffective on buttonball. Ammonium sulfamate applied as a foliage spray on black willow and trumpet vine at 50 pounds per acre gave only moderate control one year after treatment. Treatment of three woody species with these formulations as foliage sprays showed that they were relatively ineffective on a pound per acre basis as compared with 2,4-D sprays.

TABLE 9

Effectiveness of Ammonium Trichloroacetate and Ammonium Sulfamate for the Control of Various Marginal Species

Data based on analysis of 1020 quadrats. Plots treated during summer of 1947.

SPECIES AND TIME OF EVALUATION	AMMONIUM TRICHLOROACETATE 20%, 100 lbs. Active Material Per Acre — Per Cent Control				AMMONIUM SULFAMATE 25%, 50 lbs. Active Material Per Acre — Per Cent Control			
	Treated Plots		Check Plots		Treated Plots		Check Plots	
	Average	Range	Average	Range	Average	Range	Average	Range
Black Willow								
Same season	30	1 plot	3	(0–6) 4 plots	20	(0–36) 3 plots	3	(0–6) 4 plots
One year	23	1 plot	16	(0–43) 3 plots	47	(40–50) 3 plots	10	(0–43) 5 plots
Buttonball								
Same season	70	1 plot	0	4 plots	31	(11–53) 3 plots	0	2 plots
One year	5	1 plot	0	4 plots	3	(0–5) 3 plots	0	4 plots
Cattail								
Same season	43	(18–86) 5 plots	28	(12–4) 6 plots	49	1 plot	18	(12–23) 2 plots
One year	—		—		93	1 plot	95	(87–100) 4 plots
Panic Grass								
Same season	99	(99–100) 2 plots	0	1 plot	54	1 plot	0	
One year	100	(100–100) 2 plots	0	1 plot	0		0	
Trumpet Vine								
Same season	19	1 plot	1	(0–4) 3 plots	24	(10–37) 2 plots	1	(0–4) 3 plots
One year	18	1 plot	18	(0–38) 3 plots	42	(21–63) 2 plots	18	(0–38) 3 plots
Wool Grass								
Same season	70	1 plot	0	1 plot	—		—	
One year	—		—		—		—	
Lizard Tail								
Same season	—		—		13	1 plot	0	2 plots
One year	—		—		7	1 plot	4	(0–13) 4 plots

SUMMARY

1. Phytocidal studies were conducted on some 500 treatment plots in the Tennessee Valley involving the analyses of more than 8,000 sampling quadrats and field counts of more than 150,000 plants.

2. Formative effects were observed relative to roots, stems, leaves, flowers, fruits, and seeds. The formative effects appear to be induced at the time of exposure of the plant to the hormone and may be displayed by the plant a year or more after exposure depending upon the time the affected part unfolds or breaks its dormancy.

3. The volume of spray for control is not considered to be important for most species provided the same 2,4-D acid equivalent is applied and complete coverage obtained. However, high volume coarse sprays are favored in working near sensitive crop plants to minimize the drift hazard.

4. Most species studied were found to be most susceptible to 2,4-D formulations after full summer foliage was reached until fall. Cattail was a notable exception and was found to be more susceptible during its spring growth period.

5. Comparative studies of various 2,4-D formulations were made on seven species of littoral plants. Wide variations in results in replicated plots made it impossible to establish specific formulations as the limiting factor. Comparisons of all esters as a group versus all salts as a group showed that there were no significant differences between these two types of formulations for the control of black willow, trumpet vine, cocklebur, lizard tail, and cattail. There was some indication that there might be some small differences in the phytocidal properties of the individual 2,4-D formulations. However, it is felt that such differences, if real, are mainly of academic interest as variations with the same formulation were often greater than differences between formulations. On lotus, the esters appeared to be favored over the salts for actual control although both gave similar leaf kills the same season of application.

6. No practical control of buttonball was obtained with application rates up to 12 pounds acid equivalent per acre using salts, straight esters of 2,4-D, mixtures of 2,4-D and 2,4,5-T, and mixtures of 2,4-D and pentachlorophenol.

7. The use of a salt of 2,4-D is recommended for the control of plants such as black willow, trumpet vine, cocklebur, and weak rush as the salts are more economical and have the added advantage of being relatively fumeless as compared to the esters. An ester is recommended for the control of a plant such as lotus where actual control has been very erratic with salts but excellent with esters.

8. 2,4-D can be used effectively for the control of at least the following littoral species:

Black Willow (Salt or Ester)
Trumpet Vine (Salt or Ester)
Lotus (Ester, preferably)
Parrots Feather (Ester, preferably)
Water Primrose (Salt or Ester)
Hairy Water Primrose (Salt or Ester)
Water Willow Weed (Salt or Ester)
Weak Rush (Salt or Ester)
Cocklebur (Salt or Ester)
Giant Ragweed (Salt or Ester)

9. The minimal lethal dosage of 2,4-D for lotus was found to be approximately $1\frac{1}{2}$ ounces acid equivalent per acre. The minimum lethal dosage for most other susceptible species appears to be considerably higher (1 to 3 lbs. per acre).

10. Mixtures of 2,4-D and 2,4,5-T were studied on lotus and buttonball, but no striking evidence of synergism was found with these species. However, observations of upland coppice sprayed with the mixture showed that many so-called resistant woody species were killed readily. The concentration of the spray is also thought to be of importance. General observations suggest that mixtures of 2,4-D and 2,4,5-T possess a real synergistic action when applied to many woody plants.

11. The use of kerosene as a carrier appeared to offer no special advantage and in the interest of economy and facility of application, water is preferred. Sprays would be applied either as aqueous solutions or emulsions. Likewise, the addition of a wetting agent appeared to offer no special advantage.

12. Tables are given listing the susceptibility of 26 woody and 59 herbaceous species to hormone formulations.

13. It appears desirable that airplane application be limited to the off-season for cotton or to areas safely removed from cotton or other sensitive crop plants.

14. Ammonium trichloroacetate gave essentially 100 per cent control of panic grass, *Panicum agrostoides*, for at least two years after a single application of 100 pounds active material per acre. The same formulation was ineffective on buttonball. Ammonium sulfamate applied as foliage spray to black willow at 50 pounds per acre gave only moderate control one year after treatment.

RESUMEN

1. Se realizaron estudios de fitocidas en unas 500 parcelas sometidas a tratamiento en el Valle del Tennesse las cuales comprendieron el análisis de más de 8.000 cuadros de prueba con contajes de más de 150.000 plantas.

2. Efectos perjudiciales fueron observados en las raíces, tallos, hojas, frutos y semillas. Estos efectos parecen ser inducidos cuando la planta es expuesta a la hormona y pueden ser mostrados por la misma un año o más después de la exposición dependiendo del tiempo en que la parte afectada se desdobla o despierta.

3. Se considera que el volúmen del rociado no es importante para la mayoría de las especies siempre que el mismo ácido equivalente en 2,4-D sea aplicado y que se obtenga una cobertura completa. Sin embargo, se favorece el uso de rociados voluminosos gruesos para trabajos en la vecindad de plantas sensibles a objeto de evitar el peligro de acarreo por el viento.

4. La mayor parte de las especies estudiadas se encontraron más suceptibles a las fórmulas de 2,4-D desde el máximum del follaje alcanzado después del verano hasta el otoño. Españada fué una excepción notable encontrándose más suceptible durante su período de crecimiento en primavera.

5. Fueron hechos estudios comparativos de varias fórmulas de 2,4-D aplicadas a siete especies de plantas costeras. Las grandes variaciones obtenidas en los resultados hicieron imposible establecer fórmulas específicas como elementos determinantes. Comparaciones de todos los ésteres tomados como un grupo contra todas las sales tomadas como otro grupo no mostraron diferencias significantes en el control de

sauce negro, jazmín trompeta, lampazo, rabo de lagarto y espadaña. Hubo algunas muestras de que pudiera haber cierta diferencia entre las propiedades fitocidas de las diversa fórmulas individuales de 2,4-D. Sin embargo, se tiene la impresión de que si en realidad existen esas diferencias son principalmente de interés académico puesto que las variaciones con una misma fórmula fueron a menudo mayores que las encontradas en fórmulas diferentes. En el loto, los ésteres parecieron ser preferibles a las sales aunque ambos mataron las hojas en forma similar durante una aplicación en la misma estación.

6. No se obtuvo control práctico del plátano de Occidente con ratas de aplicación hasta de 12 libras de ácido equivalente por acre mediante el uso de sales, ésteres de 2,4-D y 2,4,5-T y mezclas de 2,4-D y pentaclorofenol.

7. Se recomienda el uso de una sal de 2,4-D para el control de plantas tales como sauce negro, jazmín trompeta, lampazo y junquillo, puesto que las sales son más económicas y tienen la ventaja adicional de producir menos vapores que los ésteres. Un éster en cambio, es recomendado para el control de plantas tales como el loto donde el control con sales ha sido muy irregular pero excelente con ésteres.

8. El 2,4-D puede usarse con efectividad para el control de por lo menos las siguientes plantas costeras:

Sauce Negro (Sal o Ester)
Jazmín Trompeta (Sal o Ester)
Loto (preferible Ester)
Plumaje de Loro (preferible Ester)
Primavera de Agua (Sal o Ester)
Primavera de Agua Peluda (Sal o Ester)
Maleza de Sauce Acuático (Sal o Ester)
Junquillo (Sal o Ester)
Lampazo (Sal o Ester)
Zuzón Gigante (Sal o Ester)

9. La dosis letal mínima de 2,4-D para loto fué aproximadamente de $1\frac{1}{2}$ onza de ácido equivalente por acre. La dosis letal minima para la mayor parte de las especies suceptibles fué considerablemente mayor (1 a 3 libras por acre).

10. Mezclas de 2,4 D y 2,4,5-T sobre loto y Plátano de Occidente fueron estudiadas pero no se halló ninguna evidencia resaltante de sinergismo en estas especies. Sin embargo observaciones de rociadas con esta mezcla sobre sotos evidenciaron que muchas de las especies arbóreas resistentes fueron muertas fácilmente. La concentración del líquido parece también tener importancia. Observaciones generales sugieren que las mezclas de 2,4-D y 2,4,5-T poseen una verdadera acción sinergista sobre muchas plantas leñosas.

11. El uso de kerosene como vehículo no parece ofrecer ventaja especial y en el interés de economía y facilidad de aplicación debe preferirse el agua. Los líquidos se aplicarían bien como soluciones acuosas o bien como emulsiones. La adición de un agente humedecedor tampoco parece ofrecer ventaja especial.

12. Se ofrecen tablas que muestran la suceptibilidad de 26 especies leñosas y 59 especies herbaceas a fórmulas con hormonas.

13. Es conveniente que la aplicación por avión se haga en las épocas que no haya

plantaciones de algodón o bien limitarlas a las áreas donde el algodón y otras plantas sensibles hayan sido removidas.

14. El tricloroacetato de amonio dá un control ciento por ciento del mijo (*Panicum agrostoides*) que se extiende hasta por lo menos dos años después de una sóla aplicación de 100 libras por acre. La misma fórmula resultó ineficaz contra el Plátano de Occidente. Sulfonato de Amonio rociado sobre el follaje en sauces negros a razón de 50 libras por acre dió solamente control moderado después de un año del tratamiento.

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Grateful acknowledgment is made of the help and assistance given by the many individuals who participated in planning and in conducting these studies. The late Mr. C. C. Kiker and members of the engineering staff were particularly helpful in providing advice, facilities, and assistance in carrying out the studies. The studies were also favored by the broad experience and stimulating help of Dr. W. C. Muenscher, Consultant. Special mention should be made of the help and assistance given by Robert Sparkman, James Sellers, and Arthur Morris. Airplane treatments were ably carried out by the pilots of the Authority. Thanks are also due to all members of the engineering and biology staffs for their help, assistance, and advice during various phases of the work. Representatives of various manufacturing companies were helpful and their courtesies are appreciated.

THE UNFOLDING PROGRAM OF VECTOR CONTROL IN CALIFORNIA WITH REFERENCE TO STUDIES OF MOSQUITO BIOLOGY[1]

RICHARD F. PETERS, DEED C. THURMAN, Jr., BASIL G. MARKOS and THOMAS D. MULHERN

Vector control in California has experienced a growth and expansion which is consistent with the increased knowledge of vector borne diseases gained during the past half century. The expansion has been such that even in California it should be termed "unusual". Early in the present century the late Professor W. B. Herms took the lead in malaria and mosquito control in the state and his work presaged the development of the present expanded program. Beginning with Herms' and Gray's antimalaria demonstrations, as early as 1910, the mosquito control activities in California retained this single emphasis until early in the present decade when encephalitis, previously reported in California, was proved by W. McD. Hammon and W. C. Reeves through their epidemiological findings to be an important mosquito borne disease in the State. This fact, coupled with public demand for protection against insect borne diseases which might be introduced following World War II, brought about the expansion of state and local vector control programs, particularly those directed against mosquitoes.

Unique to California, the principal mosquito vector control problems are related to the use of irrigation water in the Central Valley. About four million acres of the potential eight and one-half million irrigable acres of land are now under irrigation annually. The expansion of this type of agriculture through the Central Valley Project and related conservation and water distribution projects will add more than three and a half million acres of potential habitat to the annual mosquito problem. This vast irrigated area in the Central Valley comprises the zone of endemicity of both malaria and the arthropod-borne virus encephalitides.

In the past twenty years, a combination of some eliminative control and many repeated application of toxicants has been used. While both approaches still are essential to the present day attack, even a combination of them is sometimes deficient in providing economical mosquito control, irrespective of mosquito vector control. Eliminative measures are not always immediately feasible and are frequently financially prohibitive on a short term basis. Control through the use of toxicants offers no prospect for any reduction in future control requirements. To further complicate the problem, in California it is already apparent from reports of local agencies that larval mosquito resistance has developed to chlorinated hydrocarbons.

For all these reasons it has become increasingly necessary that we understand the basic characteristics of mosquito biology. With this understanding we shall be in a better position to improve present approaches and to develop new methods and techniques.

The history of malaria in California indicates that *Anopheles freeborni* Aitken has

[1] From the Bureau of Vector Control and the Communicable Disease Center, Public Health Service, Federal Security Agency, Atlanta, Ga.

been the chief vector of malaria, yet in the days of malaria prevalence, expansive sources of *A. freeborni* were rare. In the present decade rice culture in California has become a major agricultural industry. During 1949 approximately 300,000 acres were planted in rice, chiefly in the Sacramento Valley (northern half of the Central Valley). Rice fields have come to support a tremendous population of *A. freeborni*, larvae of which frequently occur in numbers averaging 20 per dip and higher throughout the fields at the peak of the season. Adult females of this mosquito migrate in tremendous numbers over distances which may be as great as 20 miles or more from the rice fields to hibernation shelters. Such shelters frequently are associated with human habitation around which they become severe pests, biting during warm winter days.

The fact has long been recognized that *Culex tarsalis* Coquillett is widely distributed and prevalent throughout California. This mosquito will propagate in almost all non-brackish water standing for a period of more than a week. It develops dense populations in a variety of habitats, the most important of which is rice fields, probably making it the most common mosquito found generally throughout the State. Its preference for avian blood makes it less a pest to human beings than either *Anopheles* or *Aedes*. However, as a vector mosquito for viral encephalitides, it is regarded as the most significant species in California.

Aedes mosquitoes in California, both fresh and salt water species, constitute the State's most significant control requirement. Almost all species, even those inhabiting tree holes, feed avidly on human blood. In the mountain areas, *Aedes* mosquitoes arising from snow melts plague recreational areas, while in certain coastal areas human outdoor activities are seriously interfered with by salt marsh *Aedes*. By far the most extensive and intensive *Aedes* problem is associated with irrigated land in the Central Valley. Permanent pasture of ladino clover and various forage grasses, which crop supports the dairy and beef cattle industry in the Central Valley, is the dominant source of *Aedes nigromaculis* (Ludlow) and *Aedes dorsalis* (Meigen).

Previous to 1937 *A. nigromaculis* was unknown to California; and by interesting coincidence, the first appearance of *A. nigromaculis* in the State closely approximates the beginning of extensive use of ladino clover in permanent pasture.

A. dorsalis has been recognized since the beginning of mosquito work in California as a major pest in both irrigated areas and saline marshes. It remains significant though now outnumbered by the almost unbelievable density of *A. nigromaculis*.

A. nigromaculis, *A. dorsalis*, and *A. vexans* have been shown to be efficient laboratory vectors of one or more of the arthropod-borne virus encephalitides (Madsen and Knowlton, 1935; Madsen, Knowlton, and Rowe, 1936; Herms, 1939; Hammon and Reeves, 1943). The increasing population of *Aedes* in the Central Valley of California, particularly the unusual and astonishing increases made by *A. nigromaculis* in the past 5 years, makes it desirable, if not absolutely necessary that virological studies of the relationships of these mosquitoes to the transmission of the viruses of the encephalitides in nature be further elucidated. *A. dorsalis* has been found naturally infected with encephalitis virus (Hammon and Reeves 1945). Further studies are needed to more fully establish the role of the *Aedes*, especially *A. nigromaculis* in field transmission, which does not detract in any way from the importance of the established *Culex* vector.

So serious is the *Aedes* problem to local control authorities that its reduction is

essential to public comfort and it must be undertaken before any mosquito vector control program is sanctioned by the general public during non epidemic periods. If local agencies are unsuccessful in their *Aedes* program financial support for the control of proved vector species will not be forthcoming.

The unfolding program of vector control as it relates to these problems envisages having obtained increased knowledge of the biology of the mosquitoes concerned. This demands close investigation into:

The seasonal cycle, which must be observed for several years in order to determine the prevalence of all stages through the year, the number of broods occurring each year, and the overwintering potentialities of eggs, larvae, pupae, and adults.

The distribution throughout the State of the mosquito species, as well as their relative densities and habits, which need to be ascertained in order that any spread of these mosquitoes relating to changing agriculture may be immediately known.

Mosquito ecology surveys must be made in order that knowledge of the range and limitations of larval habitats will be available. The factors influencing habitat selection and survival under optimum as well as adverse and average conditions are important to the mosquito control program.

Adult habits, especially host preferecne, flight habits, and dispersion must be determined.

All of these demand intensive year-round activities aimed at getting to know thoroughly and completely the mosquitoes which constitute problems in California, in order to determine where, when and how they may best be controlled.

In summation, the program being undertaken is a difficult one of long-term nature, which to be successful must be approached on as many fronts as possible. The urgency of the need is growing in face of the increasing densities of mosquitoes under existing conditions, the apparent resistance of mosquitoes to present day toxicants, the tremendous biotic potential of the species under consideration, the increased acreage which will be under irrigation in future years, and lastly, the increased demand on the part of the public for better control at decreased per capita expenditures.

An appropriate thought applicable to California's expanded concept of vector control was written editorially by Dr. L. L. Williams, (1949) Chief, Health Branch, Department of State, recently in the Journal of Economic Entomology in regard to preventing the introduction of vectors. He stated: "At present we depend on disinsectization of carriers and upon surveillance—a surer method might be based upon a greater knowledge of the habits of insects."

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FIELD STUDIES ON THE BIONOMICS OF *ANOPHELES ALBIMANUS*

PART I: AESTIVATION OF IMMATURE STAGES—PROGRESS REPORT[1]

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This report is the first of several which will deal with field observations on the anophelines of Puerto Rico. Our studies are concerned with several bionomical factors which possess actual or potential significance in the control of malaria and the control or eradication of *Anopheles albimanus*. Observations on aestivation are included in the study program in view of the significance of this factor in species eradication.

The possibility of the aestivation of immature stages of anophelines in the tropics and sub-tropics has been postulated but only rarely investigated. The seasonal prevalence of *A. albimanus* is such that various workers have considered this species to be one with which this phenomenon might occur. Attention has been directed principally to the ova, but larval and pupal stages have not been ignored in these speculations. Work done by Kumm (1941) in Costa Rica shows that at least at times an unusual type of ova may be found. In addition, Stone and Reynolds (1939) have reported the apparent "hibernation" of *A. albimanus* eggs in Panama. It has been suggested that these might be a resistant stage, and correlated with seasonal conditions, but this has not been confirmed.

In Puerto Rico *A. albimanus* is believed to be the most important vector of malaria and for this reason studies on its biology have been carried on for a considerable period of time. However, as far as can be determined, this aspect of its biology has never been carefully investigated. It has been noticed by some workers that, shortly after the beginning of the rains, particularly on the south coast, large populations of adults can be captured in electric light and stable traps. This occurs within the time span required from oviposition to emergence of the adults. Therefore, it has been postulated that aestivation in an immature stage might occur in Puerto Rico.

Puerto Rico is divided by a mountain range that extends practically the entire length of the island, forming a main east-west axis, which causes marked differences in both the total and seasonal precipitation occurring in the north and south coastal plains. Portions of the south coast are characterized by low annual precipitations (20 to 35 inches), more sharply defined wet and dry seasons, and a relative lack of permanently wet areas, as compared with other parts of the island. Due to these and other ecological factors favoring aestivation and because there are also extensive

[1] Studies performed under a grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service, sponsored by Columbia University School of Public Health.

quantitative records of adult anopheline populations for the region, this portion of the island was selected for these studies.

In the late fall of 1948, reconnaissance visits were made on the south coast of the island and highly productive breeding areas in various ecological situations were selected for study. It was desired to establish experimental areas in as many areas as possible with respect to water-holding characteristics, type of soil and vegetation, precipitation, general topography and local surface configuration. However, the selection of sites was moderately limited by agricultural practices and by larvicidal and residual spray programs for malaria control which were underway in some areas. Through the cooperation of the Bureau of Malaria and Insect Control of the Insular Health Department it was possible to determine the limits of larviciding projects so that no experimental sites would be effected by this type of control. Fifteen major

Fig. 1. Pasture Collecting Area

sites were selected from Maunabo on the east to beyond Guayanilla on the west, a distance of over sixty miles. Several of these stations were subdivided to take advantage of ecological differences. Stations were selected with respect to: (1) wetting and drying, (2) soil type, (3) topography and (4) vegetation. The following types of situations were studied: (1) man-made holes; (2) seepage areas supplied intermittently by water from irrigation ditches and drains; (3) small and large temporary pools, including pasture land; (4) temporary watered marsh, apparently brackish; (5) unlined ditches; and (6) streams of various types. The selection of stations over a long expanse of coast was indicated because of the marked localized character of rainfall both in total annual amount and distributional pattern.

Observations were started in the middle of November 1948 and continued at irregular intervals until late April 1949. Before the middle of December all sites had been placed under observation and by that time repeated examinations had been

made of the first selected areas. At this time all selected locations had high larval densities. From then on all stations were examined at least twice a month and usually at more frequent intervals. In late December some stations began to dry and by early April all stations except those located on permanent streams had either been intermittently dry or were nearly dry. During drying there was usually some cracking and fissuring of the soil surface to a depth of one (1) or two (2) inches, rarely up to one (1) foot. At times there was also a concentration of aquatic life, particularly predators. Fouling of the water by various types of pollution was occasionally noticed. Apparently by these latter two methods larvae were at times eliminated before the station became completely dry. As drying began, soil samples were collected from the selected sites, and these ranged from the dry margins to more moist areas that were practically saturated but which had no free surface water. In practically

FIG. 2. Sampler and Specimen Container

all cases several samples were taken at each location and additional samples were taken at irregular intervals whenever conditions warranted. In all 356 soil samples were collected. Originally the samples were collected with a trowel and were transferred to paper plates, later to paper cups. Then a sampler of the type devised by Bradley and Travis (1942), which removed 12.57 square inches, was procured, and samples were transported and kept in cardboard containers. Particular care was taken to obtain samples from all existing conditions and to collect adequate representative material from the side walls and bottoms of soil cracks and fissures.

The samples were returned to the laboratory and kept in a mosquito-free room to avoid accidental oviposition. They were covered with water from a source known to be capable of supporting *A. albimanus* but free from either eggs or larvae. Initially all samples were kept in a naturally lighted room but as the numbers under observation increased it was necessary to divide the collections and some were placed in a

storage room which had only artificial illumination. All samples were kept at existing San Juan temperatures, at which other collections of *A. albimanus* were growing satisfactorily. These samples were observed daily for signs of larva and after being kept for at least 2 weeks were checked microscopically for ova or larvae. Throughout these studies no anophelines were encountered, but *Psorophora* larvae, psychodids and chironomids were found on several occasions.

SUMMARY

A series of 356 soil samples was collected from dried areas in which breeding of *A. albinamus* had been observed previously during wetter periods. The samples were kept in the laboratory for observation after wetting. Collections were made from areas in Puerto Rico where aestivation in immature stages would be most likely to occur. No anophelines were encountered, but Psorophora larvae, psychodids and chironomids were found on several occasions.

CONCLUSION

These preliminary results would seem to indicate that no aestivation of the immature stages of *A. albimanus* occurs under these conditions. It is desired, during the coming season, to collect additional samples from a wider variety of ecological situations. Laboratory experiments on the ability of ova to withstand desiccation also will be undertaken.

RESUMEN

Se recolectó una serie de 356 muestras de suelos provenientes de áreas secas en las cuales con anterioridad, en épocas más húmedas se había observado cría de *Albimanus.* Estas muestras se conservaron en el laboratorio para observación después de humedecerlas. Las recolecciones fueron hechas en áreas de Puerto Rico donde la estivación de los estadios jóvenes era muy posible que ocurriera. No se encontraron anophelinos, pero larvas de Psorophora, de psycoodidos y chironomidos fueron hallados en varias ocasiones.

CONCLUSION

Estos resultados preliminares parecen indicar que no ocurre estivación en los estadios jóvenes de *A. albimanus* bajo las condiciones estudiadas. Es deseable que durante la próxima estación se recolecten muestras adicionales provenientes de un área más amplia en cuanto a variedad de condiciones ecológicas. Experimentos de laboratorio en cuanto a la capacidad de los huevos para resistir la desecación también deberán emprenderse.

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NOTE ON THE HOST PREFERENCES OF *ANOPHELES PSEUDOPUNCTIPENNIS*

B. E. SASSE AND L. W. HACKETT[1]

Anopheles pseudopunctipennis is the principal malaria vector in the Andean region of South America from Ecuador to Northern Chile. While its range and breeding habits are fairly well known, practically nothing has been published about its behavior as an adult and, especially, its host preferences when seeking a blood meal. Such knowledge is important not only to measure its local effectiveness as a vector but also to throw light on the probable existence of a *pseudopunctipennis* complex consisting of two or more distinct species or geographical varieties possessing different feeding habits within the immense range of this anopheline.

In coastal Peru, which lies entirely in the tropics, *A. pseudopunctipennis* occurs from sea level to about 2,600 meters, and while it is present in varying numbers throughout the year in the lower valleys, the transmission season is surprisingly short, producing epidemic malaria only for a period of three or four months annually under the most favorable conditions. The average enlarged spleen in children is small in spite of high spleen indexes which often exceed 50 per cent. All this points to a rather inefficient vector whose numbers rise above the threshold required to ensure transmission for only a short period each year.

In order to find out more about the relative attraction which human beings have for this species, a stable trap was placed in operation in April, 1948, in Palpa, a small coastal city of Peru located on the Pan American Highway some 400 kilometers south of Lima. The town lies near the River Palpa which provides favorable breeding places for the mosquito, and the trap was set up in five different locations along the river bank for varying periods of time, being operated five nights a week during 12 weeks. These locations, with one exception, were between the river and the town, and the distances from the nearest inhabited houses varied from 50 to 300 meters. The trap was placed facing west throughout the experiment and captures were made each morning at 7 o'clock by the same person. Each week on separate nights a man, goat, pig, calf and donkey was placed in the trap. The same man and animals were used throughout the study.

Although captures were low, the number caught remained about the same during the 12-week period. The only exception was when the trap was located near the highway for five days when the numbers fell considerably due probably to the presence of a number of dairy cattle nearby.

The average nightly capture for each type of bait was as follows:

1. Donkey .... 62.3 anopheles
2. Calf .... 45.5 anopheles
3. Pig .... 42.6 anopheles
4. Goat .... 42.0 anopheles
5. Man .... 33.8 anopheles

[1] The studies and observations on which this paper is based were conducted with the support and under the auspices of the International Health Division of The Rockefeller Foundation in co-operation with the Peruvian Health Ministry.

DISCUSSION

Throughout the study the trap was in close proximity to a large number of human habitations. The construction of these dwellings was such that a mosquito would have no difficulty in entering and leaving them. In fact, in many cases, the bedrooms were without doors and the cane walls often had no mud to stop the wide cracks which extended from floor to roof. Human blood was therefore easily accessible to the mosquitoes at a short distance from the trap.

It should be noted, however, that although the differences in the attraction exerted by the various animals used in this experiment are significant, it has often been demonstrated elsewhere that individuals of the same species show considerable differences among themselves in their relative attractiveness to a number of biting insects including various anophelines. Thus one of two men or of two donkeys, under identical conditions of exposure, may be attacked more frequently than the other. Further studies of *A. pseudopunctipennis* should be carried out to eliminate this factor. The relative size or exposed area of the animals used for bait is probably unimportant since the subjects are concealed by the trap and attract biting insects by their odor or other emanations.

CONCLUSIONS

Under the limitations of this experiment, the following conclusions are indicated:

1. *A. pseudopunctipennis* preferred the donkey used in this study to all the other baits, and showed the least preference for the man.
2. *A. pseudopunctipennis* will choose the common domestic animals for a blood meal even in the presence of human beings.

SUMMARY

A stable trap baited in rotation with four different animals and a man was operated nightly for 12 weeks in a malarious village of coastal Peru. *A. pseudopunctipennis* preferred any of the common domestic animals to man, and the donkey to other animals.

RESUMEN

Una trampa-establo con cebo de diferentes animales y un hombre en rotación fué operada todas las noches durante 12 semanas consecutivas en una aldea malárica de la costa del Perú. El *A. pseudopunctipennis* prefirió cualquiera de los animales domésticos comunes al hombre y el mono a todos los animales.

STUDIES IN HUMAN MALARIA

XXV. Trial of Febrifugine, an Alkaloid Obtained From *DICHROA FEBRIFUGA* Lour., Against the Chesson Strain of *PLASMODIUM VIVAX*

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Although the medicinal herb known as Ch'ang Shan has been used in China for many centuries as an antimalarial, only in recent years have serious attempts been made to determine its active principle. It is generally agreed that Ch'ang Shan is a local name for preparations made from the dried roots of *Dichroa febrifuga* Lour., an evergreen shrub indigenous to India and Southwestern China. The isolation of active alkaloids from this plant source has been described by at least three groups of investigators, working independently. Koepfli *et al.* (1947, 1949), who began work on the problem in 1943 under the auspices of the Board for Coordination of Malaria Studies, obtained two interconvertible alkaloids which they designated febrifugine and isofebrifugine. The former, which exists in two crystalline forms, was reported as having by far the greater activity against the parasites of avian malaria. Kuehl *et al.* (1948) likewise reported the isolation of two antimalarial alkaloids, I and II, from *D. febrifuga.* A third series of investigations has been reported from Chinese governmental laboratories (Jang *et al.*, 1946, 1948; Chou *et al.*, 1947, 1948). The last-named paper describes three isomeric alkaloids, tentatively called α-, β- and γ-dichroine. γ-dichroine was shown by Henderson *et al.* (1949) to be highly antimalarial against *Plasmodium lophurae* and *P. relictum.* Koepfli *et al.* (1949), attempting to reconcile these various findings, conclude that the characterizations of the materials obtained in these independent studies are in essential agreement, and that it is likely that isofebrifugine, Kuehl's alkaloid I and α-dichroine are identical, and that possibly β- and γ-dichroine and Kuehl's alkaloid II correspond to febrifugine (which is dimorphic)[1]. There seems to be general agreement on a molecular formula of $C_{16}H_{19}O_3N_3$. Present evidence indicates that febrifugine and γ-dichroine are 4-quinazolone derivatives.

When febrifugine was tested against *P. lophurae* in the duck it exhibited a quinine equivalent of 100 (tests G-5 and I-2[2]). Against blood-induced *P. gallinaceum* malaria in the chick (test A-1), the quinine equivalent was 64. Schmidt (1947) found febrifugine to be 100 times as active as quinine when tested in blood-induced *P. cynomolgi* malaria in the monkey.

[1] In a private communication (20 January 1950) Koepfli reports: "Recently a few milligrams of γ-dichroine were made available to us and simultaneous melting point determinations with our high melting crystal modification of febrifugine were carried out. Both substances, when heated at 1° per minute, melted at 156–157° (cor.,) and there was no depression in a mixed melting point determination. On the other hand, X-ray powder photographs of the two substances were not identical. Therefore the relationship of γ-dichroine to febrifugine and its high melting crystal modification is still unsettled."

[2] Techniques of tests G-5, I-2 and A-1 are described in Wiselogle (1946).

Pharmacological studies by Schmidt (1947) have shown febrifugine to be highly toxic. The acute oral LD_{50} in the white mouse was between 2.0 and 5.0 mgm./kg., or approximately 100 times that of quinine. A rhesus moneky given the drug every 8 hours survived 0.3 mgm./kg./day for 16 days. Another given 0.6 mgm./kg./day for 16 days exhibited loss of weight and anorexia. A third animal given 0.75 mgm./kg./day died on the 9th day. Hyperemia of the gastric mucosa and moderately

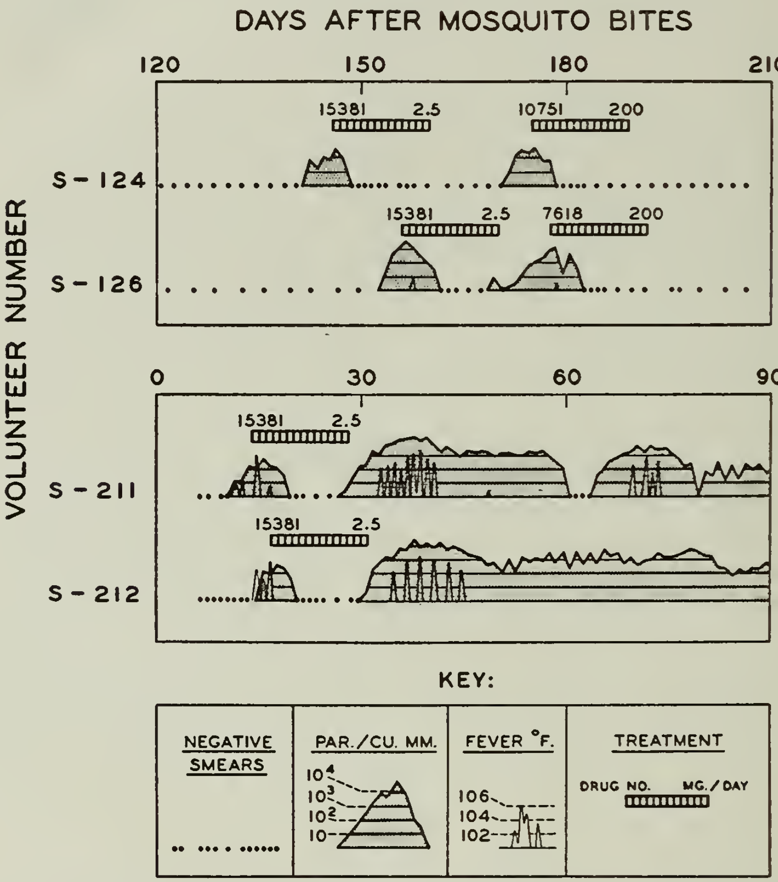

FIG. 1. Record of parasitemia and fever in four volunteers treated with febrifugine, 2.5 mg. per day for 14 days. (15,381 = febrifugine; 10,751 = camoquin; 7618 = chloroquine).

fatty liver were the only notable pathological findings. Schmidt concluded that the material was probably 300 times as toxic as quinine for the monkey, although the toxicity was qualitatively quite different.

The studies to be reported in this paper constitute a limited trial of febrifugine in man.

MATERIAL AND METHODS

Febrifugine was tested at the Federal Correctional Institution, Seagoville, Texas against six attacks of malaria in four volunteers who had been experimentally

infected with the Chesson strain of *Plasmodium vivax* by the bites of *Anopheles quadrimaculatus* mosquitoes. The testing procedure was similar to that previously described by Coatney *et al.* (1948).

During drug trials the volunteers were hospitalized and treatment was begun on the 3rd to 5th day of patent parasitemia. Thick blood smears were made and examined daily until negative for at least three successive days, when the schedule was changed to alternate days. Temperature records were made every four hours during periods of hospitalization.

The febrifugine dihydrochloride made available for these trials was supplied through the kindness of Dr. J. B. Koepfli. Data accompanying the sample are compatible with the proposed molecular formula of $C_{16}H_{19}O_3N_3 2HCl$. The corrected melting point of the base was 139–140°C., that of the salt 220–222°C. (with preliminary darkening). Dosages are expressed in terms of the base, which corresponds to 80.5 per cent of the weight of the dihydrochloride salt.

EXPERIMENTS AND RESULTS

Two volunteers (S-124 and S-126), each weighing 71 kg., were given febrifugine in dosage of 5 mgm. per day (1.25 mgm. every 6 hours) during delayed primary attacks of malaria which followed the bites of ten heavily infected mosquitoes. Due to the violent emetic action, administration of the drug had to be stopped after four doses had been given to each subject. Nausea and violent vomiting followed within two hours of each dose. Treatment was continued with other antimalarials.

When the foregoing subjects experienced their second attacks of malaria, which began 143 and 155 days respectively after exposure, febrifugine was again used for therapy, but the dosage this time was 2.5 mgm./day (0.625 mgm. every 6 hours). Nausea and frequent vomiting again occurred, but was less severe than with the higher dosage, so that the subjects were able to complete all 14 days of treatment. As shown in figure 1, blood smears became parasite-free in two to five days. Parasites reappeared in subject S-126 on the last day of therapy and in subject S-124 on the 11th day after the last dose of drug.

Two additional patients, Nos. S-211 and S-212, weighing 57 and 68 kg. respectively, were treated with febrifugine in dosage of 2.5 mgm./day for 14 days, during primary attacks. Their blood smears became negative for parasites after three to five days of therapy, and the subjects became afebrile. Again nausea and vomiting were prominent symptoms, but the men were able to complete the full courses of therapy. Parasites reappeared in the blood of each volunteer on the last full day of febrifugine therapy. These relapses were allowed to progress untreated, as shown in figure 1; parasitemia and fever persisted sufficiently long to rule out natural immunity as the cause of the initial remissions.

DISCUSSION

At a dosage of 5 mgm. per day, febrifugine given to two men with primary Chesson strain vivax malaria produced such severe nausea and vomiting that an adequate test of antimalarial potency was impossible. When given in dosage of 2.5 mgm. per day during four acute attacks, the drug, although still emetic, was sufficiently well retained for temporary clearance of parasitemia and fever. Although no direct com-

parison was made, the effect was similar to that obtained against the Chesson strain when 50 to 100 mgm. of quinine (base) are given per day. The only other drug tested by us which has shown antimalarial activity in comparably low dosage is chlorguanide, which in non-resistant strains will produce temporary clearance of patent parasitemia at a dosage of 1.5 mgm. per day (Cooper *et al.*, 1950). Febrifugine however is very poorly tolerated by the human host and its emetic action makes its use impractical. It is of interest in this connection that most references to the use of Ch'ang Shan refer to the high incidence of nausea and vomiting during the course of treatment. Despite the high potency of this active principle, its discovery remains only of theoretical interest.

SUMMARY

Febrifugine, an alkaloid derived from *Dichroa febrifuga* Lour., was shown to produce temporary alleviation in four acute attacks of experimental Chesson strain *vivax* malaria in prisoner-volunteers. Active doses, 2.5 mgm. per day (0.035–0.044 mgm./kg./day), produced nausea and vomiting in all subjects.

RESUMEN

Se demostró que febrifugina, un alcaloide derivado de *Dichroa febrifuga* Lour., produjo alivio temporal en cuatro ataques agudos de infecciones experimentales con la cepa Chesson de malaria a vivax entre prisioneros voluntarios. Dosis activas de 2.5 mgm. por día (0.035-0.044 mgm./kg./día), produjo nauseas y vómitos en todos los sujetos.

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STUDIES IN HUMAN MALARIA

XXVI. Simultaneous Infection with the Chesson and the St. Elizabeth Strains of *PLASMODIUM VIVAX*

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The suggestion has frequently been made that successive attacks of *vivax* malaria in an individual exposed in a malarious area need not necessarily be relapses caused by the same strain of parasite. It is theoretically possible for the fixed-tissue parasites of two or more strains of *Plasmodium vivax* to coexist in a host and produce malarial attacks independently. Boyd and Kitchen (1948), studying this possibility in a veteran of the Pacific campaigns of World War II, transferred parasites from successive relapses to separate recipients, and by cross-inoculation studies obtained strong evidence for the presence of two different strains. In earlier studies with experimental blood-induced infections, Boyd, Kupper, and Matthews (1938) obtained results which suggested to them that the simultaneous presence of two strains of *P. vivax* delayed the development of an adequate homologous immunity to either.

It occurred to us in 1947 that use might be made of the characteristically different relapse patterns of the Chesson and the St. Elizabeth strains of *P. vivax* to secure evidence for the existence of double-strain *vivax* infections. It will be recalled that the Chesson strain usually produces an infection with several closely-spaced attacks, whereas the St. Elizabeth strain infection exhibits an early primary attack, several months of latency and a series of late attacks in close succession (Coatney and Cooper, 1948). The study to be reported here will describe the course of events in a group of 15 volunteers, of whom nine were inoculated with the two strains simultaneously, three were inoculated with the St. Elizabeth strain alone and three with the Chesson strain alone.

MATERIAL AND METHODS

The study was carried out in white male volunteers who were inmates of the Federal Correctional Institution, Seagoville, Texas. The general procedures which were employed have been described previously by Coatney *et al.* (1948). Infections were initiated by the bites of artificially reared *Anopheles quadrimaculatus* mosquitoes which had been infected two weeks earlier by allowing them to feed upon mental patients undergoing malarial fever therapy. After the infected mosquitoes had fed upon three volunteers in turn, each insect was dissected and its salivary glands examined for the presence of sporozoites. The number of sporozoites found was graded on a scale of 1+ to 4+.

Volunteers remained under close observation for 18 months after exposure; blood smears were taken at least once weekly. All malarial attacks were interrupted by treatment with chloroquine diphosphate, 2.5 grams (1.5 grams of base) given over a four-day period, begun on or before the fifth day of patent parasitemia.

TABLE 1

Summary of activity in volunteers infected simultaneously with the St. Elizabeth and Chesson strains of Plasmodium vivax, *compared with controls given each strain separately*

Each acute attack was interrupted with the same dosage of chloroquine.

VOLUNTEER NO.	INOCULUM				ONSET PRIMARY ATTACK (DAY AFTER BITES)	TOTAL NUMBER OF ATTACKS	DAYS FROM END OF TREATMENT TO RELAPSE, BETWEEN ATTACKS				ONSET OF LAST ATTACK (DAY AFTER BITES)
	St. Elizabeth		Chesson								
	No. mosq.	Sum of pluses*	No. mosq.	Sum of pluses			1 & 2	2 & 3	3 & 4	4 & 5	
S-57	5	15	5	17	12	4	97	79	148	—	356
S-58	5	15	5	17	12	3	66	53	—	—	141
S-59	5	15	5	17	12	5	43	119	49	39	293
S-60	5	19	5	10	14	4	58	187	43	—	319
S-61	5	19	5	10	14	3	248	45	—	—	317
S-62	5	19	5	10	13	3	254	41	—	—	320
S-63	5	19	5	11	14	5	42	87	136	43	347
S-64	5	19	5	11	14	5	53	50	62	61	265
S-65	5	19	5	11	14	4	57	128	81	—	297
S-66	10	35	—	—	11	4	177	37	41	—	286
S-67	10	35	—	—	13	4	226	48	51	—	359
S-68	10	35	—	—	16	4	116	126	48	—	325
S-69	—	—	10	25	14	4	49	58	121	—	263
S-70	—	—	10	25	14	3	50	98	—	—	172
S-71	—	—	10	25	14	3	42	49	—	—	117

* The infective inoculum for each subject is expressed as the sum of the individual sporozoite densities in the mosquitoes which bit the subject.

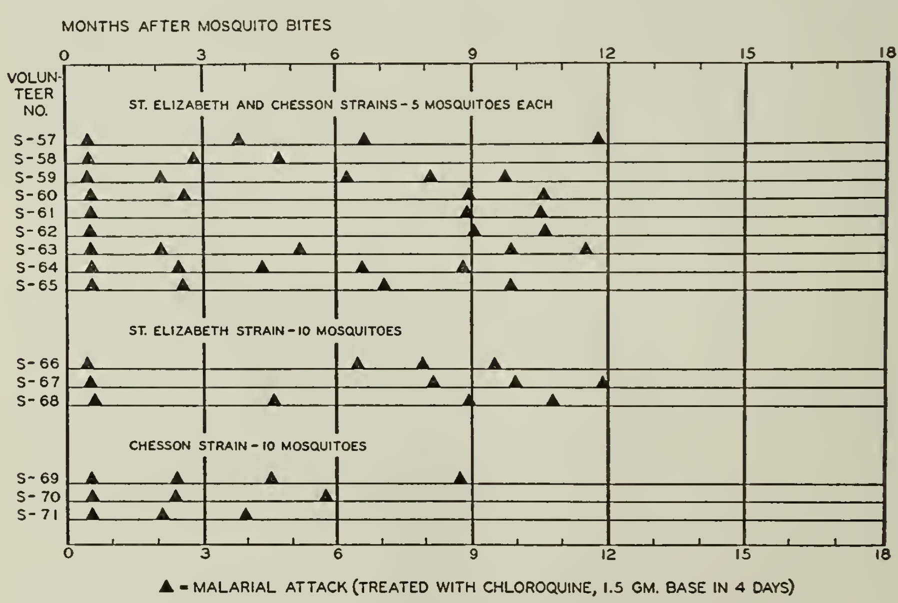

FIG. 1. Pattern of malarial attacks in volunteers S-57 through S-71 during 18 months of observation. All episodes of patent parasitemia are shown. The presentation is diagrammatic; parasitemia actually persisted in the various attacks for from three to nine days.

EXPERIMENT AND RESULTS

The fifteen volunteers were bitten by mosquitoes on 3 February 1947. As outlined in table 1, each of the first nine men was bitten by five mosquitoes infected with the St. Elizabeth strain and by five infected with the Chesson strain, a total of ten infected mosquitoes. Each of the next three was bitten by ten St. Elizabeth-infected mosquitoes, and each of the final three by ten Chesson-infected mosquitoes.

All of the fifteen volunteers had primary attacks of malaria beginning 11 to 16 days after exposure. These and subsequent attacks were interrupted with identical regimens of chloroquine. The pattern of relapses can be noted in figure 1. The St. Elizabeth strain controls had no relapses during the first trimester, one during the second trimester,[1] four during the third and four during the fourth trimester. The Chesson strain controls had three relapses during the first trimester, three during the second and one during the third. The nine doubly-infected men displayed six relapses during the first trimester, four during the second, nine during the third and eight during the fourth trimester. The latent intervals between successive attacks are detailed in table 1, as are the times of onset of the subjects' last attacks.

DISCUSSION

The St. Elizabeth strain controls displayed long latency after their primary attacks and the peak of activity was during the third and fourth trimesters, as expected (Coatney *et al.* 1950). In the Chesson strain controls, relapses occurred during the first, second and third trimesters, at the usual intervals observed when chloroquine is used for therapy. Men in the doubly-infected group, who had had the same total number of mosquito bites as the controls, displayed recurrent activity throughout a period of nearly one year, without either long latency or early termination. There were individual variations, however, for S-58 displayed a relapse pattern similar to that of the Chesson strain controls, whereas S-61 and S-62 could not be distinguished from St. Elizabeth strain controls. The collective picture, however, was that of a combination of the two strain patterns.

That the average number of acute attacks per volunteer was no greater in the doubly-infected group than in the St. Elizabeth group and only slightly greater than in the Chesson group probably reflects total sporozoite dosage per man, which was in the same range for all groups. Each man in the first group was bitten by 10 infected mosquitoes, five for each strain; each of the controls likewise was bitten by ten infected mosquitoes. The total malaria experience of the doubly-infected group would therefore not be expected to correspond to an actual addition of the patterns in the control groups.

The Chesson strain infections in this experiment, as exhibited in volunteers S-69, S-70 and S-71, were of shorter duration than is usual with this strain following the bites of 10 mosquitoes. We have observed many Chesson strain infections which have exhibited 12 or more relapses occurring over periods of 15 to 18 months. It is not necessary therefore to postulate multiple strain infections to explain the pro-

[1] This attack, which began on the 137th day after inoculation, was the earliest appearance of late activity in our entire experience with the St. Elizabeth strain, involving 198 patients (Coatney *et al.*, 1950). The only other late attack appearing in the second trimester began on day 179.

longed course of all stubbornly-relapsing cases of *vivax* malaria. In view of the lack of complete cross-protection between strains, however, it is logical to assume that if several strains were present more attacks of malaria would be required before tolerance and immunity to all strains would develop.

SUMMARY

Volunteers infected with the Chesson and St. Elizabeth strains of *Plasmodium vivax* displayed a relapse pattern consistent with a combination of the relapse patterns which were exhibited by the two strains when present separately.

RESUMEN

Voluntarios infectados con las cepas Chesson y St. Elizabeth de *Plasmodium vivax* presentaron un cuadro de recaída que consistió en una combinación de los cuadros observados separadamente en recaídas debidas a cada una de las cepas en referencia.

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WHO EXECUTIVE BOARD REQUESTS CO-ORDINATION OF RESEARCH ON ANTIMALARIALS

The Executive Board of the World Health Organization at its fifth session approved a recommendation of the Expert Committee on Malaria concerning field and hospital trials of chemotherapeutics used in the treatment and in the suppression of malaria.

The Executive Board invites the attention of the Member Governments to the benefits that might be derived in achieving co-ordination of national institutes undertaking research of this nature, such co-ordination resting on the basic principles outlined by the Committee. Member Governments are asked to request their relevant institutes to consider favorably the recommendation formulated by the Expert Committee on Malaria and either invite them to contact the headquarters of the Organization, or to furnish the list of such institutes, if it is preferred that WHO should approach them, with a view to establishing a working arrangement aiming at the co-ordination envisaged by the Expert Committee.

BASIC PRINCIPLES FOR FIELD AND HOSPITAL TRIAL OF MALARIA THERAPEUTICS AND SUPPRESSIVE TREATMENTS

The Expert Committee on Malaria of WHO favors the view that the WHO should concentrate on stimulating and co-ordinating field and hospital trials of antimalarials through existing institutions in different countries, rather than to undertake such trials under its own initiative. The Committee feels that the results expected from the latter course would involve disproportionate expenditure of money and effort which could not be justified, keeping in view the budgetary limitations of WHO. The Committee, however, considers that the WHO should use its good offices to direct these inquiries along uniform lines, so that the results obtained from the different countries would, as far as possible, be comparable. For this purpose, it would be advisable to circulate the different institutions, either direct or through the corresponding experts certain basic principles listed below, for their approval.

The results of these investigations should be obtained direct from the co-operating institutes, or through the members of the Expert Committee or the corresponding experts, and tabulated in the Malaria Section of the WHO Secretariat. The Committee does not favor the alternative procedure of collecting such information by circulating a tabulated proforma to responsible investigators and to co-operating Institutes.

CHEMOTHERAPEUTIC TRIALS

(1) Trials should be limited to microscopically diagnosed cases of malaria, and results analyzed by species of parasite.

(2) The chemotherapeutic agents should be administered only by a competent and responsible member of the investigation staff.

(3) The number of cases treated should be sufficiently large to allow of adequate appraisal.

(4) All available evidence should be given as regards history of previous attacks of malaria or exposure to infection.

(5) The trials of a new drug should be run against simultaneous tests with a known drug.

(6) The effectiveness of the test drug should be measured by the following criteria:

(a) subsidence of fever below 100°F; recorded in 12 hour periods after the first dose;

(b) disappearance of parasitemia; recorded in days after the first dose;

(c) the interval between the end of treatment and reappearance of parasites and/or fever should be determined. The follow-up should preferably extend over a period of one year and statement should be given as to whether the subject has been exposed to re-infection.

(7) The epidemiological features of the area where the malaria cases under trial acquired the infection should be considered, with particular reference to:

(a) the endemicity of malaria;

(b) whether or not any anti-mosquito measures were in force, and with what success;

(c) transmission season;

(d) the degree of severity of the malaria season in the investigation period as compared with previous years.

SUPPRESSIVE TREATMENT TRIALS

(1) The chemotherapeutic agent must be administered only by a competent and responsible member of the investigation staff, who should maintain a register wherein the necessary entry is made against each individual by name *at the time the drug is administered.*

(2) The population under trial should be as stable as possible and sufficiently large to allow of adequate appraisal.

(3) All suspected break-throughs should be confirmed by microscopic diagnosis. They should be treated with the same drug as that used for suppression.

(4) The area selected for trial should be highly endemic or hyperendemic.

(5) The trial of a new drug should preferably be run against simultaneous tests with a known drug in approximately equal groups of persons living in the same locality.

(6) The epidemiological features of the area should be recorded, with particular reference to:

(a) the endemicity of malaria;

(b) whether or not any anti-mosquito measures were in force, and with what success;

(c) transmission season;

(d) the degree of severity of the malaria season in the investigation period as compared with previous years.

OVERSEAS ASSIGNMENTS AVAILABLE

To meet the increasing demand for experienced health personnel to staff technical health missions overseas which have been authorized by Congress, the Division of International Health, Public Health Service, is developing an intensive recruiting program.

Opportunities for overseas assignments in the higher grades are expected to develop for a number of physicians, scientists, health educators, sanitary engineers, sanitarians, nurss, administrators, and technicians. Some of the projects will involve employment by the Public Health Service and some will involve employment by the World Health Organization.

Members of technical health missions can assist foreign governments in establishing public health training, initiate health demonstrations, supervise specific projects, and serve in an advisory capacity to foreign government officials on health matters.

The various overseas health missions of the United States have been authorized by Congress with a view to strengthening mutual understanding between the people of the United States and the people of other countries. Such missions offer a challenge to American health experts to cooperate with the other people of the world in the development of human resources, as well as an opportunity to broaden their own medical and personal horizons.

Recruitment will be limited to highly qualified personnel possessing both expert knowledge in their technical specialties and the ability to inspire cooperation in a constructive program directed toward broad improvements in public health and the general advancement of human relationships.

Assignment will be made in the higher grades. Additional compensation will be provided in the form of allowances for overseas service.

Qualified health personnel may obtain application forms and further details concerning opportunities to participate in these programs by writing to the Chief, Division of International Health, Public Health Service, Federal Security Agency, Washington 25, D. C.